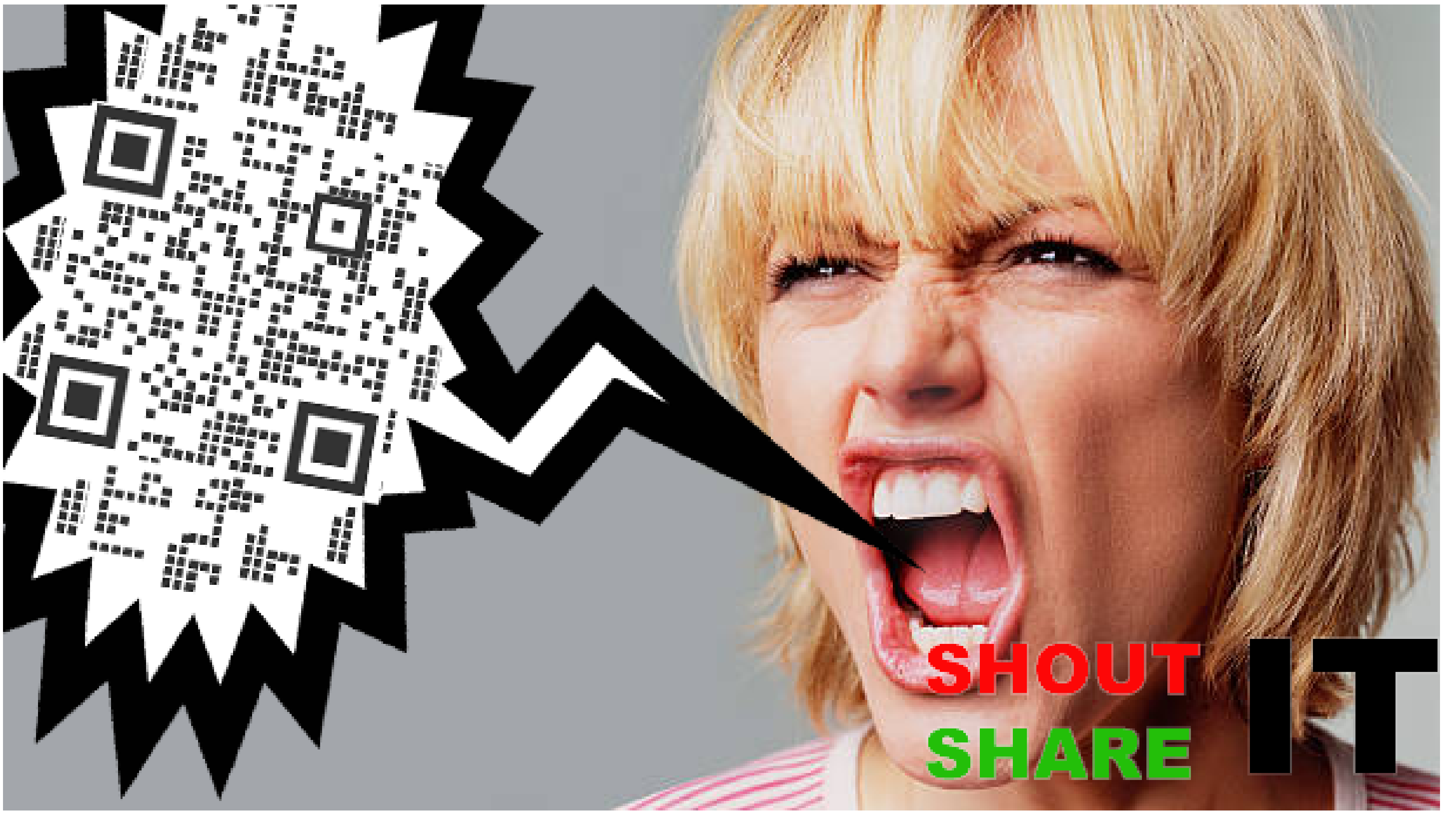
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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:00 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 9:00 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

The financial situation of the Laboratory at the end of 1950 was about the same as it was in December, 1949. Total cash receipts for the year amounted to $327,185.65 and expenditures, including $17,514.05 for capital assets, were $324,383.96. These totals were $19,550.51 and $14,283.03, respectively, in excess of the totals for 1949. The increases in charges for tuition, laboratory space and dormitory and Mess accommodations were just about sufficient to enable the Laboratory to meet increasing costs including a few very much needed salary and wage adjustments, but the margin is much too slim. Additional income for current operating expenses is still of the utmost urgency.

The statement of actual cash receipts and disbursements which has been given you in recent years is believed to be helpful in giving the overall picture, but in the case of some of the departments listed it may be misleading because of the fact that it does not include shares of the overhead cash expenses grouped under administration, buildings and grounds, etc. For instance, if overhead expense and depreciation are included, the Mess had a deficit of $2,145.52 and the dormitories a deficit of $1,258.68. Conversely, in the case of the Supply Department, when the cost of specimens furnished to Research and Instruction (estimated at $16,368.20) and other bookkeeping items are taken into account, there is a net income of $1,379.84 instead of the loss of $9,462.61 on a departmental cash basis.

Income from the Endowment Funds of the Laboratory increased $6,084.44 over last year to $46,431.59, due to larger common stock dividends. The average yield on securities was 4.8% on book value and 4.3% on market value. The investments and percentages in the Endowment Funds as of Jan. 1, 1951, were:

	Book Value	%	Market Value	%
Bonds				
U. S. Government	$367,997.66	38.31%	$ 361,447.87	33.90%
Railroad	54,613.02	5.65	59,662.50	5.60
Public Utility	90,192.50	9.34	89,706.25	8.41
Industrial	34,800.00	3.60	34,283.75	3.21
Total Bonds	$549,603.18	56.90	$ 545,100.37	51.12
Preferred Stocks	125,828.41	13.03	123,877.25	11.62
Common Stocks	290,371.21	30.07	397,386.25	37.26
Total Securities	$965,802.80	100.00%	$1,066,363.87	100.00%
Principal Cash	2,419.51		2,419.51	
Totals	$968,222.31		$1,068,783.38	

The Balance Sheet, Statement of Current Surplus, and Summary of Cash Transactions, as given in the report of the auditors, Seamans, Stetson and Tuttle of Boston, follow:

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DEC. 31, 1950

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee		$ 968,222.31	
Securities and Cash in Minor Funds		18,998.47	$ 987,220.78
Plant Assets:			
Land	$ 113,626.38		
Buildings	1,490,666.08		
Equipment	257,161.69		
Library	403,433.74		
	$2,264,887.89		
Less Reserve for Depreciation	786,616.68	$1,478,269.21	
Book Fund Cash		2,939.75	$1,481,208.96
Current Assets:			
Cash		$ 22,924.33	
Mortgage Note Receivable		2,350.00	
Accounts Receivable		19,874.97	
Inventories:			
Supply Department	$ 53,670.83		
"Biological Bulletin"	16,191.21	69,862.04	
Investments:			
Devil's Lane Property	35,648.04		
Stock in General Biological Supply House	12,700.00		
Other Investment Securities	46,109.29		
Retirement Fund	20,244.36	114,701.69	
Prepaid Insurance		7,056.77	
Items in Suspense (Debits)		1,161.41	$ 237,931.21
			$2,706,360.95

Liabilities

Endowment Funds:			
Endowment Funds	$ 966,445.89		
Reserve for Amortization	1,776.42	$ 968,222.31	
Minor Funds		18,998.47	$ 987,220.78
Plant Funds:			
Mortgage Notes Payable		$ 11,000.00	
Donations and Gifts	$1,331,699.04		
Other Investments in Plant from Gifts and Current Funds	138,509.92	1,470,208.96	$1,481,208.96
Current Liabilities and Surplus:			
Accounts Payable		$ 5,361.61	
Items in Suspense (Credits)		$ 1,055.00	
Current Surplus		231,514.60	$ 237,931.21
			$2,706,360.95

Current Surplus Account

Balance, January 1, 1950		$ 215,443.60
Add:		
Excess of Income over Expense for Year	$ 4,685.77	
Reserve for Depreciation, 1950, Charged to Plant Funds	29,160.29	33,846.06
		$ 249,289.66
Deduct:		
Payments from Current Funds during year for Plant Assets:		
Buildings	$ 779.45	
Equipment	5,773.72	
Library	10,221.89	
	$ 16,775.06	
Payment on Principal of Mortgage Note given in Partial Payment for "Dolphin"	1,000.00	17,775.06
Balance December 31, 1950		$ 231,514.60

Summary of Cash Transactions for Year ended December 31, 1950

		Expenditures		
Current Cash	Receipts	Current	Additions to Capital Assets	Total Expenditures
Income from Endowment Funds	$ 46,431.59			
Income from Other Investments	22,502.00			
Real Estate Rentals	6,864.64	$ 1,781.74		$ 1,781.74
Donations	24,532.51			
Membership Dues	2,460.00			
Instruction	16,811.15	10,073.86		10,073.86
Research (including Apparatus and Chemical Departments)	30,738.61	26,949.50	$ 2,929.06	29,878.56
Supply Department	74,079.32	82,896.48	645.45	83,541.93
Mess	48,513.37	47,123.02	964.50	48,087.52
Dormitories	22,824.85	10,541.46	467.96	11,009.42
"Biological Bulletin"	7,551.71	14,253.77		14,253.77
Library	4,974.91	8,591.71	9,874.48	18,466.19
Frank R. Lillie Memorial Fund—Arbacia Survey	5,129.49	4,235.87		4,235.87
Office of Naval Research	3,522.50	3,440.16		3,440.16
Miscellaneous	4,576.07	755.73		755.73
Book Purchase Account, Woods Hole Oceanographic Institution	800.00	838.79		838.79
Real Estate Sales (on account)	4,872.93			
Administration		24,718.91		24,718.91
Buildings and Grounds		51,010.98	1,632.60	52,643.58
Interest, Insurance and other Property Maintenance		10,769.50		10,769.50
Payments to Retirement Fund		8,888.43		8,888.43
Payment on Mortgage			1,000.00	1,000.00
	$327,185.65	$306,869.91	$17,514.05	$324,383.96

Cash Balance, January 1, 1950:		
Central Hanover Bank and Trust Co.	$ 8,159.11	
Falmouth National Bank	2,131.69	$ 10,290.80
Receipts		327,185.65
		$337,476.45
Payments		324,383.96
Cash Balance, December 31, 1950		
Central Hanover Bank and Trust Co.	$ 11,003.27	
Falmouth National Bank	2,089.22	$13,092.49

Other Cash Accounts	Balance Jan. 1, 1950	Received	Paid	Balance Dec. 31, 1950
Allen R. Memhard Fund Cash	$ 58.05	$ 25.00		$ 83.05
Rev. Arsenious Boyer Burse Cash	561.33	125.00	$ 504.46	182.07
Lucretia Crocker Fund Cash	1,747.88	440.43	1,513.39	674.92
Bathing Beach Fund Cash	1,000.00			1,000.00
Dr. Frank R. Lillie Memorial Fund Cash	4,952.47	18,598.15	15,218.78	8,331.84
Old Main Building Fund Cash (Rockefeller)	30,720.64		30,720.64	
Retirement Fund Cash	4,555.05	11,082.29	14,637.99	999.35
Bio Club Scholarship Fund Cash	130.03	62.50	120.00	72.53
Book Fund Cash	838.93	5,056.25	2,601.70	3,293.48
Lalor Foundation Fellowships Cash	2,118.21	5,000.00	5,057.28	2,060.93

Respectfully submitted,
Donald M. Brodie,
Treasurer

V. REPORT OF THE LIBRARIAN

The sum of $12,544 was appropriated to the Library for the year 1950, plus $3000 from the Woods Hole Oceanographic Institution to be applied to staff salaries. This amount was expended as follows: serials, $4427.38; books, $565.17; binding, $2100.87; supplies and sundries, $843.37; freight and express, $41.61; insurance, $55.00; and salaries, $7768.89. The total expenditures amounted to $15,802.29.

In addition to the above, $800 (plus a balance of $63.89 from 1949) was received from the Woods Hole Oceanographic Institution for acquisitions covering the field of oceanography. Of this amount, $804.79 was spent, leaving a balance of $59.10 on Dec. 31, 1950.

From the Carnegie Corporation of New York Fund, 24 completed back sets and 37 partially completed back sets were purchased, at a cost of $3078.49. The balance of this fund will be used during the coming year and a final summary of acquisitions realized from the $25,000 will be given in the 1951 report.

During the year, 1478 (66 new) current journals were received. Of these, 480 (17 new) were Marine Biological Laboratory subscriptions, 587 (24 new) were exchanges and 158 (13 new) were gifts; 69 (3 new) were Woods Hole Oceanographic Institution subscriptions; 161 (3 new) were exchange and 23 (6 new) were gifts.

The Marine Biological Laboratory purchased 66 books, received 5 complimentary copies from authors, 85 titles from publishing firms and 21 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 6 titles, making a total of 183 books added to the shelves.

There were 27 back sets completed: 26 by purchase (2, Woods Hole Oceanographic Institution), and 1 by exchange; 51 were partially completed: 42 by purchase (5, Woods Hole Oceanographic Institution), 1 by exchange and 8 by gift.

The reprint additions to the Library numbered 5146. Of these, 2009 were of current issue and 3137 of earlier dates. Acknowledgment is made to Dr. Dorothy Wrinch and to Dr. A. M. Pappenheimer for gifts of reprints totalling 4780. Of these, 1299 were not already contained in the Library's collection.

Fifty volumes were borrowed on inter-library loan and 125 were sent to other institutions. Eighty-four microfilm orders were filled, amounting to $190.53.

The amount of $172.49 was realized from the sales room, and from this sum, new lights for the cataloguing room have been purchased.

There were two changes made in the staff during the year—Miss Benevides and Miss Hoffer joined the personnel, replacing Mrs. McInnes and Miss Goodfellow.

At the end of the year 1950, the Library contained 59,980 bound volumes and 174,081 reprints.

For the ever-willing help received from the Library Committee during 1950, the Librarian wishes to express her appreciation.

Respectfully submitted,
Deborah Lawrence Harlow,
Librarian

VI. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

The sixty-third year of the Marine Biological Laboratory opened with a new administrative leadership and organizational set-up. The retirement of Dr. Charles Packard and of Mr. F. M. MacNaught, effective at the close of the preceding year, created serious gaps in the direction and management of the Laboratory. Fortunately we had available for the position of General Manager, Mr. Homer P. Smith who had had the valuable experience of serving as Assistant Business Manager with Mr. MacNaught.

Mr. Smith took over his duties as General Manager with energy and imagination. With the excellent cooperation of the Maintenance staff, he has continued the rehabilitation of the Laboratory plant initiated by Dr. Packard at the close of the war. Mr. Smith with the assistance of Mr. R. W. Kahler, has developed a long-range plan which, if it can be carried out in the present emergency and in the future, will result in the maintenance of our plant in first-rate condition.

The reorganization of the Laboratory administrative set-up, developed by a committee under the chairmanship of Dr. Eric G. Ball, has worked most satisfactorily. The lines of authority and responsibility are well defined, resulting in excellent cooperation and performance by the members of the permanent staff.

Mess Hall Management

During the winter Mr. Smith explored the possibilities of employing a new steward for the Mess Hall. Miss Belle Downing completed this season fifty years of summer service in the Mess Hall, the past several years in direct charge. These duties, combined with the supervision of the dormitory maid service, were too heavy for one person and Mr. Robert T. Martin, Manager of the dining hall at Harvard University's Adams House, took over as steward. He proved a most fortunate choice as Mr. Martin, assisted by Mr. John De Petris, has done an outstanding job. Increased cost of food and help necessitated an increase in Mess rates which was more than compensated by the improvement in the food and service.

Contributions and Gifts

Contributions to the Frank R. Lillie Memorial Fund during the year totaled $25,860.12. The Laboratory is much indebted to the contributors which included the Eli Lilly Company, $15,000; Dr. G. H. A. Clowes, $5,000; the Kettering Foundation, $2500; and the General Biological Supply House, $1000. A number of the Corporation members also contributed and it is hoped that other members will also give to the fund.

Contributions from the Associates totaled $1465, and, in addition, there was an anonymous contribution from one of the associates who donated $1000 to the Laboratory to be used as was deemed most advisable. With the Associates' contribution, the Laboratory has purchased a steam generator which has been urgently needed by the Laboratory for the past several years.

Dr. William Curtis, as has been his custom in the past, made a special donation to the Laboratory: this year, a handsome compass and an automatic heater for the *Dolphin.*

Grants

The American Philosophical Society has instituted a program through which they support three research rooms annually for the next five years. This grant amounts to $1800 each year and covers the total maintenance costs of these rooms. Already this has proved a real asset, providing space for visiting foreign investigators who frequently do not have funds available to cover the Laboratory charges.

The American Cancer Society continued its support of the "hot lab" by a grant which will provide experienced technical assistance so necessary in the handling of radioactive materials.

The Laboratory continues to receive support from the Rockefeller Foundation from a grant which has three more years to run. These funds supplement the regular Laboratory income for general operations and are very important in helping the Laboratory operate with reasonable effectiveness. This past summer we enjoyed a visit from President Chester I. Barnard of the Rockefeller Foundation and Dr. Warren Weaver, Director of the Natural Science Division. They spent two days surveying the research and teaching activities and facilities of the Laboratory. They were impressed by the need of the Laboratory for certain new items of equipment and apparatus.

Lalor Fellowship Program

Dr. C. Lalor Burdick visited the Laboratory in August, talked with the Lalor Fellows and met with the Fellowship Committee. Dr. Burdick encouraged the Committee to apply for a renewal of the fellowship grant and this was done. Notice has been received from the Foundation that the fellowship grant has been renewed for five years and increased to $6000 annually. The outstanding success of this program should encourage the Laboratory to seek additional funds for fellowships in fields not covered by the Lalor Grant.

Office of Naval Research Contract

A contract with the O. N. R. provided funds in the amount of $5000 for the collection, transportation, and distribution of squid for research purposes. A truck was especially equipped with a cooled, aerated, salt water tank. With this equipment, it has been possible to transport squid from the north side of the Cape to Woods Hole, the animals arriving at the Laboratory in excellent condition. The funds also provided a new engine for the *Tern*, which brings in squid from the Martha's Vineyard Sound fish traps.

Arbacia Program

There was a serious shortage of Arbacia for research purposes during the 1949 season. With funds from the Frank R. Lillie Memorial Fund, an Arbacia scouting project was developed in 1950, to locate new collecting beds. Dr. John S. Rankin spent the major part of the summer dredging likely areas and located a long, narrow bed off the northwest shore of Martha's Vineyard between Gay Head and Menimsha Bight. It is hoped that in the near future an ecological program may be developed for the investigation of Arbacia. Through the cooperative use of the Arbacia collected from the old beds, most investigators obtained adequate materials. Some investigators substituted for their research other forms which, in some cases, were more satisfactory than Arbacia.

Ecology Program

Early in the year a committee was set up under the Chairmanship of Dr. W. C. Allee to develop an ecological program. Such a program appeared desirable, particularly in view of our difficulty in obtaining some forms in adequate numbers for research and, still more, to broaden the base of operation of the Laboratory. As a first step the Committee recommended the establishment of a course in ecology at the graduate level for a limited number of students. This was approved by the Board of Trustees, provided funds could be obtained to support the course. The problem was discussed with Mr. Fairfield Osborn, President of the New York Zoological Society. This Society very generously has made a grant of $4000 a year for three years to assist us in getting this program under way.

Oceanographic Institution Expansion

There is urgent need for expansion of the Oceanographic Institution plant to take care of Navy contract research resulting from the present emergency. The most favorable site for this expansion is on the Bar Neck Property adjacent to the Oceanographic Institution. This property is owned by the Marine Biological

Laboratory. A majority of the M. B. L. Trustees oppose the sale of this unique piece of property, although there was considerable support for leasing the property. It should be possible to solve this problem to the mutual satisfaction of both institutions, permitting effective expansion of the Oceanographic Institution but, at the same time, protecting the interests of the Marine Biological Laboratory.

Biological Bulletin

At the termination of this year, Dr. Donald P. Costello became editor of the "Biological Bulletin," succeeding Dr. H. Burr Steinbach.

Housing

Housing continues to be a vexing problem though it has been eased somewhat by the building of several houses in the Devil's Lane Tract during the past two years. The roads in this tract have been accepted by the town and power lines and water mains have been installed.

There are appended as parts of this report:

1. Memorial
2. The Staff
3. Investigators and Students
4. The Lalor Fellows
5. Tabular View of Attendance
6. Subscribing and Cooperating Institutions
7. Evening Lectures
8. Shorter Scientific Papers Presented at the Seminar
9. Members of the Corporation

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
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MASS.

Respectfully submitted,
PHILIP B. ARMSTRONG,
Director

1. MEMORIAL

Leonor Michaelis

By

E. S. G. BARRON

The Marine Biological Laboratory mourns the death of Professor Michaelis. The loss is more deeply felt, as his death came just two months after the Department of Physiology had decided to celebrate his 75th birthday by dedicating to him the Physiology Course of 1950. He was very touched by this tribute and had accepted our invitation to spend the summer with us. Students and staff expected to profit from his genius and his wisdom. A member of the Corporation since 1928, Professor Michaelis was an instructor of the Physiology Course for ten years.

Born in Berlin, Germany, on January 16, 1875, Leonor Michaelis graduated from the Medical School in Berlin in 1897. That his life was to be devoted to the pursuit of knowledge was demonstrated early. While still a student he published a paper on the histology of milk secretion which was rewarded with a prize from the Medical School, the only recognition that was to be awarded to him in time. In Professor Hertwig's laboratory young Michaelis worked on embryological problems; his paper on the cytology of the fertilization of the ovum of the amphibian *Triton taeniatus*, as well as his doctor's thesis on the determination of the direction of the first cleavage in the frog's egg, reveals rare insight and clarity of conception.

It was natural that Michaelis' early scientific steps were directed toward microscopic anatomy, since medical thinking at that time was strongly influenced by the rapid advances made with the aid of the microscope. Perhaps he would have directed himself toward pathology if, "by some strange coincidence" as he says it, he had not met Paul Ehrlich, who at that time was looking for a young histologist. Ehrlich offered him a one-year position as his private assistant in the State Institute for Serological Testing and Research. It was here that Michaelis studied the chemistry of staining, and it was here that the vital staining of mitochondria with Janus Green originated. There is no doubt that this one year at Ehrlich's laboratory exerted a decisive influence on the direction of his investigations. The whole field of physico-chemical sciences as applied to biology and medicine was here opened to him. With his powerful and quick intellect he visualized the unlimited applications of physics and chemistry to biological phenomena. The science of Biochemistry at the start of the century, when Michaelis entered Ehrlich's laboratory, was still in its infancy, mostly sheltered in physiological and pathological laboratories; and the *Journal of Biological Chemistry, Biochemical Journal* and *Biochemische Zeitschrift* did not appear until six years later. Here, in front of him, was a field of vast possibilities, and he with no financial facilities, and no formal, classical training to enter it. Indeed Ehrlich himself had advised him to enter clinical medicine, which he had to do, becoming an assistant to Professor Litten in a department of one of the municipal hospitals in Berlin from 1900 to 1904. A herculean task was this, taking care of the sick and at the same time preparing himself with the solid foundations required for fundamental research in quantitative biology. With no teachers to guide him, armed only with his genius and his tenacious perseverance, he studied chemistry, as he once told me, in a dictionary, and mastered the branches of mathematics necessary to become familiar with the solution of physicochemical problems. And while thus schooling himself for the task ahead, he still found time to continue his investigations in histology and staining.

In 1903 he was made a *Privadozent* at the University of Berlin with a thesis on immunological protein precipitins. By combining enzyme techniques he observed that short digestion of serum albumin with pepsin changed profoundly the immunological properties. He was also the first to show that aqueous extracts of normal livers could be used for the Wassermann test, and that the complement fixation test in the Wassermann reaction could be replaced by a direct precipitation test by using extracts of syphilitic foetus of high potency.

In 1904, a paper about some iron-containing inclusions in tumors of the urinary bladder aroused the interest of Professor v. Leyden who appointed him research assistant for the newly created Institute for Cancer Research at the University Clinic. He worked here on experimental cancer, and once was sent to Jena to bring the ultramicroscope newly built by Siedenhopf and Zsigmondy at the Zeiss optical works. With this instrument v. Leyden had great hopes of finding the cause of cancer; Michaelis had great hopes of utilizing the instrument for studies of colloidal chemistry (which he indeed succeeded in doing).

In 1905, Michaelis received the title of Professor. What could a Professor do with no salary, no laboratory, no funds to support his work? Fully realizing the hopelessness of this situation, he accepted the position of bacteriologist at the municipal hospital "am Urban" in Berlin, which brought him in contact with Peter Rona, the chemist of the hospital. The close friendship and association with Rona lasted throughout his seventeenyear stay at the hospital. It was here that the full powers of Michaelis came to glorious eclosion. In his humble research room, with barely space enough—as Albert Szent-Györgyi has said to me—for one man to walk through, and with scarce equipment, Michaelis accomplished, either alone or in collaboration with his friend Peter Rona and a number of young scientists from abroad and from Germany, the series of remarkable investigations on the ionic environment, on weak electrolytes, on adsorption, on proteins, on enzymes—chapters which are now the secure foundation of quantitative biology.

Simultaneously with Sørensen, Michaelis foresaw the influence of the hydrogen ion concentration on biological phenomena and proceeded to develop the theory and practice of hydrogen ions. The hydrogen electrode he built for exact measurements. The theory of buffers, or, as he called them, "hydrogen ion regulators," was lucidly presented. The theories of the dissociation of amphotheric electrolytes, and of the iso-electric points of amino acids and proteins were developed quantitatively, after Hardy had introduced in a qualitative way the concept of the isoelectric point. He developed the method of electrophoresis in such a way as to keep the pH constant during the flow of current. The isoelectric points of casein, hemoglobin, serum albumin, serum globulin, native and denatured globin, gelatine, and edestin were thus determined. He was the first to show that the charge of a colloid, enzyme, or even of whole bacteria was not a property inherent in the substance alone but was dependent upon the pH and that there is an isoelectric point characteristic of the substance.

He was devoting his attention at the same time to the field of enzymes. Mindful of the influence of ionic environment, he studied the effect of the hydrogen ion concentration on enzyme activity, and although Sørensen published first similar studies, Michaelis proceeded to extend Sørensen's observations by showing that the dependence of enzyme activity on pH was of the same nature as the dependence of the dissociation of a weak acid on pH. By comparing the enzymatic activity at constant pH but varied concentrations of substrate he presented lucidly and quantitatively the concept of affinity constant. And thus the theory of enzyme-substrate combination, postulated by Henry in 1903, was established on a firm basis ten years later, in 1913. At a time when the protein nature of enzymes was as yet unknown, Michaelis guided by his genius foresaw it, and with daring and his elegant simplicity put forth the theory of enzyme-substrate complex formation. Now the Michaelis enzyme-substrate complex is one of the solid foundations of enzymology.

In a study of the effect of adsorption on enzyme activity he gave evidence of site specificity on the enzyme surface. And in his studies on the nature of enzyme inhibitors he distinguished the competitive inhibitors from those which influence the rate of splitting of the enzyme-substrate into the split products and free enzyme.

Studies on adsorption, on bacterial agglutination, on blood sugar, on buffers, and on pH indicators crowded those years of intense production.

The international reputation of Michaelis was widespread. In 1921 he received the title of *professor extraordinarius* of physical chemistry applied to medicine and biology at the University of Berlin. Again a professorship with no salary, no laboratory, no budget. Grim irony this "appointment" must have seemed to him! Hence, he accepted in 1921 the position of consultant in an industrial concern for scientific apparatus where he was provided with laboratory facilities. Less than one year later, however, he was invited to the chair of biochemistry at the re-organized medical school of Nagoya. Three pleasant and fruitful years were spent in Japan, where he initiated, in collaboration with Fujita, his studies on membrane permeability. Struck by the selective permeability of cell membranes, he strove to construct a model which would show similar properties, and thus he made his dried collodium membranes permeable only to cations. Here again he gave proof of his rare insight, for he terminated these studies upon recognizing that his models did not reproduce the properties of cellular membranes.

During his stay in Japan, Michaelis received an invitation from Jacques Loeb for a lecture tour in the United States, to be given in the summer of 1924. On the very day of his departure from Japan he received the tragic news of Jacques Loeb's sudden death. The lectures, however, had all been properly arranged, and on that occasion Michaelis was asked to come to Johns Hopkins as a resident lecturer for three years, beginning in the Spring of 1926 at the termination of his contract in Japan.

And thus, at Hopkins, started the third cycle of Michaelis' achievements. The field of oxidation- reduction processes and of heavy metal catalysis now received most of his attention. Here, as in the field of enzymes, his genius brought forth theories of fundamental importance for the understanding of the kinetics of biological oxidations. The simultaneous discovery with Elema, from Holland, of the two-step reduction of pyocyanine in acid solutions was the starting point of Michaelis' principle of monovalent oxidation-reductions and the intermediary formation of free radicals during the oxidation process of systems which perform the reaction by transfer of two electrons. The presence of such free radicals, existing in equilibrium with their "parent substances" even in aqueous solutions, was a daring concept which appeared absurd to conservative chemists. Like his theory of enzyme-substrate formation, it was more the foresight of a genius than a methodical and secure approach. The first papers on the theory of two-step oxidations were rejected by both the *Journal of Biological Chemistry* and the *Journal of the American Chemical Society*. So indignant was one referee of the latter journal that in horror he wrote, "In short, a principle of modern scientific philosophy is violated." The paper was published in *Biochemische Zeitschrift*. Michaelis felt deeply this attack. To answer the criticisms of lack of conclusive evidence and of too much reliance on potentiometric data, he diligently started the study of quantum mechanics and of magnetochemistry, attacking them with the same enthusiasm as when thirty years previously he had prepared himself in organic chemistry and mathematics. He simplified the method of the magnetic balance for the measurement of the paramagnetism of these intermediate compounds and left no room for doubt about the existence of free radicals. Thus, the name of Michaelis was incorporated in every modern text book on organic chemistry.

Yet official recognition came always late. He became a member of the National Academy of Science five years after his retirement as active member of the Rockefeller Institute, where he spent the last years of his life—from the end of his lectureship at Johns Hopkins until his death in October of 1949.

With the death of Michaelis we lose a man of an intellectual stature that is more and more rare in this age of narrow specialization. He had the priceless gift of transforming the most abstruse and complex concepts into simple and clear ideas; the books he wrote were models of clarity and conciseness. His textbook on embryology reached nine editions. *Hydrogen Ion Concentrations, Manual of Physical Chemistry, Introduction to Mathematics for Biologists and Chemists* and *Oxidation-reduction Potentials* were all translated into various languages. His brilliant genius allowed him to perceive immediately the important points in any discussion, and his wide knowledge in the fields of biology, medicine, chemistry, physics, and mathematics, was always at the service of those who went to him for advice.

The humanistic type of Gymnasium where Michaelis received his secondary education broadened his intellectual training and interests. With no difficulties in Latin or Greek he tackled the Japanese language when in Japan and became quite a connoisseur of the old Oriental civilizations. Music attracted him greatly. In his first semester at the University he even took a course in counterpoint with Professor Bellerman. His improvisations at the piano were always a delight to his listeners.

The Marine Biological Laboratory mourns deeply the loss of a great man of science and a scholar of broad interests, as well as a friend of many years' standing.

2. The Staff, 1950

Philip B. Armstrong, Director, State University of New York, Medical Center at Syracuse.

Senior Staff of Investigation

E. G. Conklin, Professor of Zoology, *Emeritus,* Princeton University.
W. E. Garrey, Professor of Physiology, *Emeritus,* Vanderbilt University Medical School.
R. S. Lillie, Professor of General Physiology, *Emeritus,* The University of Chicago.
A. P. Mathews, Professor of Biochemistry, *Emeritus,* University of Cincinnati.
G. H. Parker, Professor of Zoology, *Emeritus,* Harvard University.

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University.
Libbie H. Hyman, American Museum of Natural History.
A. C. Redfield, Woods Hole Oceanographic Institution.

II. Instructors

L. H. Kleinholz, Associate Professor of Biology, Reed College, in charge of course.
W. D. Burbanck, Professor of Biology, Drury College.
C. G. Goodchild, Professor of Biology, S. W. Missouri State College.
John H. Lochhead, Assistant Professor of Zoology, University of Vermont.
Madelene E. Pierce, Associate Professor of Zoology, Vassar College.
W. M. Reid, Professor of Biology, Monmouth College.
T. H. Waterman, Assistant Professor in Biology, Yale University.
Marion H. Pettibone, Arctic Research Laboratory, U. S. National Museum.

III. Laboratory Assistants

Robert S. Howard, Northwestern University.
Muriel Sandeen, Northwestern University.

EMBRYOLOGY

I. Instructors

Donald P. Costello, Kenan Professor of Zoology, University of North Carolina, in charge of course.
Arthur L. Colwin, Assistant Professor of Zoology, Queens College.
Charles B. Metz, Assistant Professor of Zoology, Yale University.
James A. Miller, Professor of Anatomy, Emory University.
S. Meryl Rose, Associate Professor of Zoology, University of Illinois.
Albert Tyler, Associate Professor of Embryology, California Institute of Technology.

II. Research Assistant

Margaret E. Davidson, McGill University.

III. Laboratory Assistants

J. Bruce Guyselman, Northwestern University.
Donald E. Kent, University of North Carolina.

PHYSIOLOGY

I. Consultants

Eric G. Ball, Professor of Biochemistry, Harvard University Medical School.
Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania.
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine.
Arthur K. Parpart, Professor of Biology, Princeton University.

II. Instructors

E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago, in charge of course.
Daniel Mazia, Professor of Zoology, University of Missouri.
Hans Neurath, Professor of Biochemistry, Duke University Medical School.
John Runnström, Director of the Wenner Grens Institute, Stockholm.
H. Burr Steinbach, Professor of Zoology, University of Minnesota.
George Wald, Professor of Biology, Harvard University.

III. Laboratory Assistant

Patricia McIntyre, Johns Hopkins Medical School.

BOTANY

I. Consultants

Bostwick H. Ketchum, Woods Hole Oceanographic Institution.
Wm. Randolph Taylor, Professor of Botany, University of Michigan.

II. Instructors

Maxwell S. Doty, Assistant Professor of Biology, Northwestern University, in charge of course.
George J. Hollenberg, Professor of Biology, University of Redlands.
R. D. Wood, Assistant Professor of Botany, Rhode Island State College.

III. Laboratory Assistants

Ralph A. Lewin, Yale University.
Cecilia Rieger, Vassar College.

IV. Collector

Hannah T. Croasdale, Research Associate, Dartmouth College.

V. Lecturers

J. B. Lackey, Medical and Scientific Editor, The Blakiston Company.
Ruth Patrick, Curator of Limnology, Academy of Natural Sciences of Philadelphia.

EXPERIMENTAL RADIOLOGY

G. Failla, College of Physicians and Surgeons, Columbia University.
L. Robinson Hyde, Phillips Exeter Academy, Exeter, N. H.

LIBRARY

Mrs. Deborah Lawrence Harlow, Librarian
Mary A. Rohan
Loretta J. Benevides
Joan M. Hoffer

APPARATUS DEPARTMENT

Robert Mills, Manager
James D. Graham
James D. Graham, Jr.
Elizabeth J. Keeler
Seaver R. Harlow

CHEMICAL DEPARTMENT

Gail M. Cavanaugh, Chemical Supplies Manager

SUPPLY DEPARTMENT

James McInnis, Manager
John S. Rankin, Naturalist
Ruth Crowell
Janice McLaughlin
M. B. Gray
A. M. Hilton
W. E. Kahler
G. Lehy
Carl O. Schweidenback
R. O. Lehy
James Whitcomb
H. S. Wagstaff
Robert Perry

GENERAL OFFICE

Homer P. Smith, General Manager
Polly L. Crowell, Office Manager
Mrs. Lila S. Myers
Miss Mary M. McLaughlin
Mrs. Norma Bonito

GENERAL MAINTENANCE

R. W. Kahler, Superintendent
Robert Adams
Richard Alberts
Robert Gunning
John H. Head
James S. Thayer
Albert Neal
Alton J. Pierce
T. E. Tawell
George A. Kahler

3. Investigators and Students

Independent Investigators, 1950

Abrams, Richard, Assistant Professor, Inst. Radiobiology and Biophysics, University of Chicago.
Allen, M. Jean, Assistant Professor of Zoology, University of New Hampshire.
Alscher, Ruth P., Assistant Professor, Manhattanville College of the Sacred Heart.
Armstrong, Philip B., Professor of Anatomy, State College of New York, Medical Center, Syracuse, N. Y.
Atwood, Kimball C., Visiting Professor of Zoology, Columbia University.
Baily, Norman A., Research Scientist, Columbia University.
Baker, Gladys E., Chairman, Plant Science Department, Vassar College.
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School.
Barnett, Robert C., Graduate Student in Physiology, University of Chicago.
Barron, E. S. Guzman, Professor of Biochemistry, University of Chicago.
Belford, Julius, Assistant Professor of Pharmacology, Brooklyn 2, New York.

Berger, Charles A., Director, Biological Laboratory, Fordham University.
Bernstein, Maurice H., Graduate Assistant, Washington University.
Bliss, Alfred F., Associate Professor of Physiology, Tufts College Medical School.
Blum, Harold F., Physiologist, Princeton University.
Boettiger, Edward C., Assistant Professor of Zoology, University of Connecticut.
Bonner, John Tyler, Assistant Professor, Princeton University.
von Borstel, Robert C., Assistant Instructor, University of Pennsylvania.
Bridgman, Anna Josephine, Associate Professor of Biology, Agnes Scott College.
Brooks, Matilda M., Research Associate in Physiology, University of California.
Bronk, Detlev, President, Johns Hopkins University.
Brown, Frank A., Jr., Professor and Chairman Biological Sciences, Northwestern University.
Brunst, V. V., Research Associate, University of Maryland Medical School.
Brust, Manfred, Research Assistant in Physiology, University of Chicago.
Bubieniec, Ernest J., Graduate Student, University of Wisconsin.
Burbanck, W. D., Chairman, Department of Biology, Drury College.
Camber, Bernard, Fellow of the Rockefeller Institute for Medical Research.
Chambers, Edward S., New York University, Washington Square College.
Chambers, Robert, Professor *Emeritus,* New York University, Washington Square College.
Charles, Donald R., Chairman, Division of Biological Sciences, University of Rochester.
Chase, Aurin M., Associate Professor of Biology, Princeton University.
Cheney, Ralph Holt, Professor of Biology, Brooklyn College.
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School.
Clark, Arnold M., Assistant Professor of Biology, University of Delaware.
Clement, A. C., Associate Professor of Biology, Emory University.
Clowes, G. H. A., Research Director, *Emeritus,* Eli Lilly and Company.
Cohen, Seymour Stanley, Associate Professor of Physiological Chemistry, University of Pennsylvania.
Colwin, Arthur L., Assistant Professor of Biology, Queens College.
Colwin, Laura N., Queens College.
Conklin, Edwin G., Professor of Biology *Emeritus,* Princeton University.
Cooperstein, Sherwin J., Instructor of Anatomy, Western Reserve University.
Copeland, D. Eugene, Assistant Professor of Biology, Brown University.
Cornman, Ivor, Assistant Research Professor, George Washington University.
Costello, Donald Paul, Kenan Professor of Zoology, University of North Carolina.
Coyle, Elizabeth E., Associate Professor of Biology, College of Wooster.
Crowell, Sears, Assistant Professor of Zoology, Indiana University.
Curtis, Howard J., Professor of Physiology, Vanderbilt University School of Medicine.
Curtis, W. C., Professor of Zoology *Emeritus,* University of Missouri.
DeFalco, Ralph, Associate Professor of Zoology, Rutgers University.
Doty, Maxwell S., Associate Professor of Biology, Northwestern University.
Elliott, Alfred M., Assistant Professor of Zoology, University of Michigan.
Fauré-Fremiet, Emmanuel, Professor, Collège de France.
Finkelstein, Paul, AEC Postdoctoral Fellow, University of Chicago.
Foster, Richard W., Assistant Curator of Mollusks, Museum of Comparative Zoology, Harvard University.
Gaffron, Hans, Associate Professor of Biochemistry, University of Chicago.
Garrey, Walter Eugene, Professor of Physiology *Emeritus,* Vanderbilt University School of Medicine.
Gasvoda, Betty, Junior Biochemist, Argonne National Laboratory.
Ghosh, Barendra N., Fellow of the Muscle Research, Woods Hole, Mass.
Gilman, Lauren C., Associate Professor of Zoology, University of Miami.
Glaser, Otto, E. S. Harkness Professor *Emeritus,* Amherst College.
Goodchild, C. G., Professor of Zoology, Southwest Missouri State College.
Gray, I. E., Professor of Zoology, Duke University.
Green, James W., Assistant Professor of Physiology, Rutgers University.
Grosch, Daniel S., Assistant Professor of Zoology, North Carolina State College.
Grundfest, Harry, Associate Professor of Neurology, Columbia University.
Hajdu, Stephen, Associate Professor, Institute for Muscle Research.
Harding, Clifford V., Research Fellow, University of Pennsylvania.

Harvey, Ethel Browne, Independent Investigator of Biology, Princeton University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Haywood, Charlotte, Professor of Physiology, Mount Holyoke College.
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania.
Henley, Catherine, Research Associate, University of North Carolina.
Hodes, Robert, Professor of Neurophysiology, Tulane University.
Holtfreter, Johannes, Professor of Zoology, University of Rochester.
Inoué, Shinya, Graduate Student, Princeton University.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Jacobs, William P., Assistant Professor of Biology, Princeton University.
Jenkins, George B., Professor of Anatomy *Emeritus,* George Washington University.
Karush, Fred, Visiting Scholar, Columbia University.
Keeffe, Mary M., Assistant Professor of Biology, College of St. Thomas.
Kelly, Sally M., Assistant Professor of Plant Science, Vassar College.
Kempton, Rudolf T., Professor of Zoology, Vassar College.
King, John W., Professor of Physiology, Morgan State College.
Kisch, Bruno, Professor of Chemistry, Yeshiva University.
Kleinholz, Lewis H., Associate Professor of Biology, Reed College.
Klotz, Irving M., Associate Professor of Chemistry and Biology, Northwestern University.
Krahl, Maurice E., Associate Professor of Biological Chemistry, Washington University, School of Medicine.
Kreezer, George L., Associate Professor, Washington University.
Kuffler, Stephen W., Associate Professor, Wilmer Institute.
Kuntz, Eloise, Instructor, Vassar College.
Lajtha, Abel, Assistant Professor, Institute for Muscle Research.
Lane, Charles E., Research Associate, University of Miami.
Lazarow, Arnold, Associate Professor of Anatomy, Western Reserve University.
Levy, Milton, Associate Professor of Chemistry, New York University College of Medicine.
Lillie, Ralph S., Professor of Physiology *Emeritus,* University of Chicago.
Lochhead, John H., Associate Professor of Zoology, University of Vermont.
Lovelace, Roberta, Adjunct Professor in Biology, University of South Carolina.
Lucké, Balduin, Professor of Pathology, University of Pennsylvania.
Marshak, Alfred, Adjunct Associate Professor, New York University College of Medicine.
Marsland, Douglas, Professor of Biology, New York University, Washington Square College.
Marmont, George, Assistant Professor of Physiology, University of Chicago.
Mazia, Daniel, Professor of Zoology, University of Missouri.
Mehler, Alan H., Research Associate, University of Chicago.
Metz, Charles B., Assistant Professor of Zoology, Yale University.
Metz, Charles W., Chairman, Department of Zoology, University of Pennsylvania.
Mihályi, Elemer, Research Assistant, Institute for Muscle Research.
Miller, James A., Professor of Anatomy, Emory University.
Millott, Norman, Professor of Zoology, University College of West Indies.
Monroy, Alberto, Visiting Investigator, Zoological Station, Naples, Italy.
Moore, George M., Chairman, Department of Zoology, University of New Hampshire.
Nachmansohn, David, Assistant Professor of Neurology, Columbia University.
Nelson, Leonard, Instructor in Zoology, University of Nebraska.
Neuberg, Carl, Visiting Professor, Polytechnic Institute of Brooklyn.
Neurath, Hans, Professor of Physical Biochemistry, Duke University.
Nicoll, Paul Andrew, Associate Professor of Physiology, Indiana University.
Palay, Sanford L., Instructor in Anatomy, Yale University, School of Medicine.
Parmenter, C. L., Professor of Zoology, University of Pennsylvania.
Parpart, Arthur K., Chairman, Department of Biology, Princeton University.
Pettibone, Marian H., Researcher, Arctic Research Laboratory, U. S. National Museum, Washington, D. C.
Pierce, Madelene E., Associate Professor of Zoology, Vassar College.
Plough, H. H., Professor of Biology, Amherst College.
Prosser, C. Ladd, Professor of Physiology, University of Illinois.
Redfield, Robert R., AEC Post-Doctorate Fellow, University of Chicago.
Reid, W. Malcolm, Head of Department of Biology, Monmouth College.

Reischer, Helen S., Research Associate, Haskins Laboratories.
Ricca, Renato A., Clinical Assistant, Hartford Hospital.
Rich, Alexander, Research Fellow in Chemistry, California Institute of Technology.
Rieser, Peter, Instructor in Physiology, Fordham University.
Ronkin, R. R., Assistant Professor of Physiology, University of Delaware.
Rossi, Harold H., Assistant Professor of Radiology, Columbia University.
Roth, Jay S., Assistant Professor of Biochemistry, Rutgers University.
Rothenberg, M. A., Research Associate, University of Chicago.
Rudenberg, F. Hermann, Graduate Student, University of Chicago.
Runnström, J., Director, Wenner-Grens Institut, Stockholm, Sweden.
Rutstein, David D., Professor of Preventive Medicine, Harvard Medical School.
Sandow, Alexander, Associate Professor of Biology, Washington Square College.
Sarkar, Nirmal K., Research Assistant, Institute for Muscle Research.
Schaeffer, Asa A., Professor of Biology, Temple University.
Schmitt, Francis O., Head, Department of Biology, Massachusetts Institute of Technology.
Schmitt, Otto H., Professor of Zoology and Physics, University of Minnesota.
Scott, Allan C., Associate Professor of Biology, Union College.
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College.
Scott, George T., Associate Professor of Zoology, Oberlin College.
Seaman, Gerald R., Instructor in Biological Chemistry, School of Medicine, Creighton University.
Shanes, A. M., Associate Professor of Physiology and Biophysics, Georgetown University, School of Medicine.
Sichel, F., Professor of Physiology and Biophysics, University of Vermont, College of Medicine.
Speidel, Carl C., Professor of Anatomy, University of Virginia.
Spratt, Nelson T., Associate Professor of Zoology, University of Minnesota.
Steinbach, H. Burr, Professor of Zoology, University of Minnesota.
Stern, Kurt G., Adjunct Professor of Biochemistry, Polytechnic Institute of Brooklyn.
Stokey, Alma G., Professor of Plant Science *Emeritus*, Mount Holyoke College.
Stoudt, Harry N., Assistant Professor of Biology, Temple University.
Straus, William L., Associate Professor of Anatomy, Johns Hopkins University School of Medicine.
Stunkard, Horace W., Professor of Biology, New York University.
Sturtevant, A. H., Professor of Genetics, California Institute of Technology.
Szent-Györgyi, Albert, Institute for Advanced Studies, Princeton.
Szent-Györgyi, Andrew, Institute for Muscle Research.
Taylor, William Randolph, Professor of Botany, University of Michigan.
Tobias, Julian M., Assistant Professor of Physiology, University of Chicago.
Tracy, Henry C., Professor of Anatomy, University of Kansas.
Trinkaus, J. P., Instructor in Zoology, Yale University.
Truant, Aldo P., Assistant Professor of Pharmacology, Tufts College Medical School.
Tyler, Albert, Associate Professor of Embryology, California Institute of Technology.
Varga, Laszlo, Institute for Muscle Research.
Villee, Claude A., Jr., Assistant Professor of Biological Chemistry, Harvard Medical School.
Vincent, Walter S., Jr., Atomic Energy Commission Fellow, University of Pennsylvania.
Vishniac, Wolf, Research Associate, New York University Bellevue Medical Center.
Warner, Robert C., Assistant Professor of Chemistry, New York University College of Medicine.
Whiting, P. W., Professor of Zoology, University of Pennsylvania.
Wichterman, Ralph, Associate Professor of Biology, Temple University.
Wilber, Charles G., Director, Biological Laboratories, St. Louis University.
Wilder, Violet M., Assistant Professor of Biochemistry, University of Nebraska.
Wilson, Walter L., Instructor in Physiology and Biophysics, University of Vermont, College of Medicine.
Wood, Richard, Assistant Professor of Botany, Rhode Island State College.
Woodward, Arthur A., Jr., Assistant Professor of Zoology, Washington University.
Wrinch, Dorothy, Lecturer in Physics, Smith College.
Zorzoli, Anita, Assistant Professor of Physiology, Washington University School of Dentistry.

Beginning Investigators, 1950

Aaronson, Sheldon, Research Associate, Haskins Laboratories.
Allen, Robert Day, Assistant Instructor in Zoology, University of Pennsylvania.
Breen, Moira, Assistant in Physiology, Vassar College.
Brummett, Anna Ruth, Fellow in Biology, Bryn Mawr College.
Cantor, Nancy Joan, University of Vermont, College of Medicine.
Clark, Marguerite R., Graduate Fellow, St. Louis University.
Colón, Doris E., Graduate Student, University of Pennsylvania.
Edgecombe, Carolyn A., Student, University of Connecticut.
Essner, Edward S., Assistant Instructor, University of Pennsylvania.
Gagnon, André, Assistant Instructor, University of Pennsylvania.
Goldstein, Lester, Graduate Student, University of Pennsylvania.
Grimm, Madelon R., Graduate Student in Botany, Cornell University.
Gross, Paul Randolph, Graduate Student, University of Pennsylvania.
Halaban, Atida, Graduate Student, University of Pennsylvania.
Harding, Drusilla, Research Assistant, University of Pennsylvania.
Hastings, John Woodland, Graduate Student, Princeton University.
Hay, Elizabeth D., Medical Student, Johns Hopkins Medical School.
Hill, Ruth F., AEC Fellow in Biophysics, Columbia University.
Hodge, Alan John, Research Assistant, Massachusetts Institute of Technology.
James, Thomas W., University of Missouri.
Honegger, Carol M., Instructor, Temple University.
Huebschman, Charles A., Graduate Student, Columbia University.
Katz, Michael, Graduate Student, University of Pennsylvania.
Kelly, John W., Graduate Student, University of Pennsylvania.
Krause, Leonard A., Student Investigator, University of Connecticut.
Malamed, Sasha, Assistant Instructor, University of Pennsylvania.
McCulloch, David, AEC Fellow in Biology, Massachusetts Institute of Technology.
McIntyre, Patricia A., Johns Hopkins Medical School.
Meyer, Hertha, Head of Tissue Culture Lab., Institute of Biophysics of University of Brazil.
Michelini, Francis J., Research Fellow, University of Delaware.
Mitchell, Constance Jane, University of Delaware.
Neill, William A., Student, Amherst College.
Passano, Leonard Magruder, Laboratory Assistant, Yale University.
Proctor, Nathaniel K., Student, University of Pennsylvania.
Rusk, Malcolm L., Graduate Student in Zoology, Indiana University.
San Pietro, Anthony, Graduate Student, Columbia University.
Sclufer, Evelyn, Graduate Student, Bryn Mawr College.
Slane, Gertrude M., Graduate Student, St. Louis University.
Stewart, Peter A., Teaching Assistant, University of Minnesota.
Stieglitz, Alice Anne, Student, University of Pennsylvania.
Sullivan, Robert James, Instructor, Fordham University.
Tweedell, Kenyon S., Teaching Assistant, University of Illinois.
Weber, Patricia R., Graduate Fellow, St. Louis University.

Research Assistants, 1950

Allen, Frank L., University of Chicago.
Allaman, Loren E., Northwestern University.
Anderson, Norman G., Duke University.
Braganca, Beatriz M., McGill University.
Bryan, Eliza R., Columbia University.
Bryan, George C., Columbia University.
Chatterjee, J., Washington University Medical School.
Currier, Jeanne, Vassar College.
Curtis, Paul, Oberlin College.
Davidson, Margaret Elizabeth M., McGill University.
DeKornfeld, Thomas J., Georgetown Medical School.

Fass, Jerome S., Rockefeller Institute for Medical Research.
Fingerman, Milton, Northwestern University.
Fitch, Frank W., University of Chicago.
Flesher, James W., University of Chicago.
Foley, Mary T., Yale University.
Gelewitz, Edmund W., Northwestern University.
Glass, Richard, Princeton University.
Gross, Samson R., Columbia University.
Guyselman, J. Bruce, Northwestern University.
Howard, Robert Stearns, Northwestern University.
Hulfish, Barbara, University of Rochester.
Jacob, Miriam I., Rockefeller Institute.
Joseph, Rosalie, Columbia University.
Kaplan, Ann Esther, University of Pennsylvania.
Kisliuk, Roy, Columbia University.
Landau, Joseph Victor, New York University, Washington Square College.
LeBaron, George I., Jr., Union College.
Leonard, Lawrence M., Haverford College.
Lichtenstein, Robert, Harvard Medical School.
Litt, Mortimer, University of Rochester Medical School.
McIntyre, Jane H., Bryn Mawr College.
Mitchell, Ruth, Columbia University.
Moe, Robert, Fordham University.
Nathanson, Neal, Harvard Medical School.
Orski, Barbara M., Harvard Medical School.
Padawer, Jacques, New York University.
Prescott, David M., Yale University.
Rosen, Saul W., Northwestern University.
Sandeen, Muriel, Northwestern University.
Schneider, Lillian K., Columbia University.
Steinberg, Maynard, Polytechnic Institute of Brooklyn.
Stephens, Grover C., Northwestern University.
Tamm, Olga, American Geographical Society of New York.
Tanenbaum, Stuart, Columbia University.
Tietze, Frank, Duke University.
Webb, H. Marguerite, Northwestern University.
Wilson, Irwin B., Columbia University.
Wycik, Edna, Peter Bent Brigham Hospital.

Library Readers, 1950

Bartlett, James H., Professor of Physics, University of Illinois.
Bevelander, Gerrit, Professor of Histology, New York University.
Bodansky, Oscar, Head, Clinical Biochemistry, Memorial Hospital.
Burian, Richard, Retired Professor of Physiology, 262 Kent Street, Brookline, Mass.
Cantoni, G. L., Associate Professor in Pharmacology, Western Reserve University.
Case, James F., Graduate Student, Johns Hopkins University.
Cavalieri, Liebe F., Research Associate, Sloan-Kettering Institute.
Clark, Eliot R., Professor of Anatomy *Emeritus,* University of Pennsylvania.
Csepel, Martha de, Laboratory Technician, Rockefeller Institute for Medical Research.
DeLamater, Edward D., Associate Research Professor, University of Pennsylvania.
Eichel, Bertram, Assistant Research Specialist, Rutgers University.
Eichel, Herbert J., Teaching Assistant in Physiology, Rutgers University.
Fries, E. F. B., Assistant Professor, The City College of New York.
Friedler, Gladys, Graduate Student, University of Pennsylvania.
Froehlich, Alfred, Associate, May Institute for Medical Research.
Freund, Jules, Public Health Research Institute of the City of New York.
Gabriel, Mordecai, Assistant Professor of Biology, Brooklyn College.

Goff, Richard A., Assistant Professor of Zoology, University of Oklahoma.
Gould, Harley N., Professor of Biology, Newcomb College of Tulane University.
Gudernatsch, Frederick, 41 Fifth Avenue, New York 3, New York.
Gurewich, Vladimir, Assistant Visiting Physician, Bellevue Hospital.
Heroux, Olivier, Graduate Student, Laval University.
Hughes, Arthur, Research Fellow, Strangeways Research Laboratory, Cambridge, England.
Kaan, Helen W., Research Associate, National Research Council.
Kabat, Elvin A., Associate Professor of Bacteriology, Columbia University.
Keosian, John, Professor of Biology, Rutgers University.
Krasnow, Frances, Head, Department of Research, The Guggenheim Dental Foundation.
Kunitz, Moses, Member, Rockefeller Institute for Medical Research.
Lansing, Albert I., Washington University, School of Medicine.
Levine, Celia, Graduate Student, Columbia University.
Ling, Gilbert, Instructor, Johns Hopkins Medical School.
Loewi, Otto, Research Professor, New York University College of Medicine.
McCabe, Marcia, Mount Holyoke College.
McDonald, Sister Elizabeth Seton, College of Mount St. Joseph, Ohio.
Nadeau, L. V., Graduate Student in Philosophy, College of St. Thomas Aquinas.
Person, Philip, Research Associate, Rutgers University.
Quastel, J. H., Professor of Biochemistry, McGill University.
Reiner, John R., Senior Enzyme Chemist, Columbia University.
Rosenthal, Theodore B., Research Associate, Washington University.
Rowell, L. S., Associate Professor in Zoology, University of Vermont.
Roys, Chester, University of Iowa.
Schallek, William B., Hoffmann-LaRoche, Inc.
Sulkin, S. Edward, Southwestern Medical School, University of Texas.
Taussky, H. H., Research Fellow, Cornell Medical College.
Wainio, Walter W., Associate Research Specialist, Rutgers University.
Weinstein, Marvin J., Research Assistant, Squibb Institute for Medical Research.
Yntema, Chester L., Professor of Anatomy, State University of New York, Medical Center.
Wood, Darwin L., AEC Fellow, Ohio State University.

Students, 1950

BOTANY

Barnes, Albert M., Bowdoin College.
Davis, Luretta, Student, Harvard-Radcliffe.
Federbush, Sylvia, The City College of New York.
Hansen, Jørgen B., University of Copenhagen.
James, Jacqueline, Student, Vassar College.
Johnson, Nancy G., Teaching Assistant, University of Wisconsin.
Keyes, Carolyn, Wheaton College.
Krauss, Robert W., Graduate Assistant, University of Maryland.
LeMesurier, Margaret G., Student, McGill University.
Milstrey, Roger A., Drew University.
Niedergang, Ethel, Graduate Student, Brooklyn College.
Powers, Ann W., Wellesley College.
Ross, Elizabeth G., Elmira College.
Silberger, Julius, Student, Harvard College.
Strout, Phyllis M., Student, Goucher College.

EMBRYOLOGY

Anderson, Irene L., Graduate Student, Brown University.
Babbott, Elizabeth, Student, Connecticut College.
Bernstein, Paul W., Instructor of Biology, Wesleyan University.
Carpé, Lestra M., Sarah Lawrence College.
Dunnebacke, Thelma H., Instructor, Smith College.
Feightner, Lawrence E., University of Illinois.

Fowler, Ira, Student, Louisiana State University.
Gamero-Reyes, Alonso, Instructor of Zoology, Instituto Pedagogico, Venezuela.
Gravett, Howard L., Associate Professor, Texas A. and M.
Greengard, Paul, Student, Johns Hopkins University.
Jacobson, Eugene D., Undergraduate, Wesleyan University.
Konigsberg, Irwin R., Junior Instructor, Johns Hopkins University.
Lansche, James M., Student, Washington University.
Leavitt, Earle E., Instructor, Norwich University.
Lindberg, Robert G., Teaching Assistant, University of California, Los Angeles.
Mabel, Judith, Goucher College.
Magner, Ardys, Duke University.
McCullough, Kirk W., Instructor, Washington and Jefferson College.
McKibben, Juliet, Assistant Professor of Biology, Carnegie Institute of Technology.
Neff, Ruth H., Instructor, University of Missouri.
Pepper, Max P., Amherst College.
Renzi, Alfred A., Research Assistant, Syracuse University.
Sexton, Owen J., Oberlin College.
Small, Jean Elizabeth, Student, University of Massachusetts.
Smithberg, Morris, Graduate Student, University of Rochester.
Spiroff, Boris E. N., Graduate Teaching Assistant, Northwestern University.
van Breemen, Verne L., Research Assistant, State University of Iowa.
Vogel, Philip H., Graduate Student, Loyola University.
Volz, Ruth, Drew University.

PHYSIOLOGY

Baird, Joan Audrey, Graduate Assistant, Syracuse University.
Burlington, Harold, Graduate Assistant, Syracuse University.
Chin, Mary B., Teaching Fellow, University of Michigan.
Ehrmann, Robert L., Johns Hopkins University Medical School.
Friedman, Stanley, Graduate Student, Johns Hopkins University.
Gladner, Jules A., Research Assistant, Duke University School of Medicine.
Herbert, Edward, Student, University of Pennsylvania.
Karplus, Martin, Student, Harvard College.
Keller, John G. J., Graduate Fellow, St. Louis University.
Kominz, David R., Post-doctoral Fellow, Harvard University.
Lilien, Otto M., Jefferson Medical College.
Marshall, Gerald W., University of California, Los Angeles.
Medill, Mary A., University of Pennsylvania.
McMaster, Rachel D., Graduate Student, Columbia University.
Miller, Daniel G., Rockefeller Institute.
Miller, Tilford D., Amherst College.
Neff, Robert J., Research Associate, University of Missouri.
Neukom, Betty Jane, Chemical Technician, University of Colorado Medical Center.
Praglin, Julius, Assistant, University of Illinois.
Reichert, Erida R., Johns Hopkins Medical School.
Robinson, Charles V., Research Associate, Harvard Medical School.
Schneider, Rose Mary, Graduate Student, New York University.
Shaw, Evelyn S., Instructor, American Museum of Natural History.
Stallworthy, Wilson B., Instructor in Zoology, University of Maine.
Strominger, Jack L., Washington Medical School.
Townes, Philip L., Graduate Student, University of Rochester.
deVillafranca, George W., Student, Yale University.

INVERTEBRATE ZOOLOGY

Anderson, Everett, Graduate Student, Fisk University.
Anderson, Janet L., Teaching Assistant, University of Michigan.
Belmonte, Rev. Roch G., Instructor in Biology, Fordham University.
Berne, Bernard, Assistant Instructor, University of Illinois.
Bernhardt, Eva G., Associate Professor of Biology, Huntington College.

Birkner, Marion L., University of Illinois.
Bowman, Marjorie C., Graduate Student, University of Toronto.
Campbell, Mildred A., Smith College.
Conover, Robert J., Oberlin College.
Cory, B. Lawrence, Graduate Student, Notre Dame University.
Cotter, William B., Jr., Graduate Student, Wesleyan University.
Dossel, William E., Graduate Assistant, Marquette University.
Ede, Donald A., Assistant, Northwestern University.
Erk, Frank C., Johns Hopkins University.
Etges, Frank J., Graduate Assistant, New York University.
Forsthoefel, Rev. Paulinus F., Graduate Student, Ohio State University.
Gustafson, John A., Assistant, Cornell University.
Haddow, Suzanne, Student, Skidmore College.
Hodge, Mary H., Wilson College.
Hugghins, Ernest J., Teaching Assistant, University of Illinois.
Hunter, Robert L., University of Michigan.
Hyland, Kerwin E., Jr., Graduate Student, Duke University.
Imbrie, John, Graduate Student, Yale University.
Jenerick, Howard P., Public Health Service Fellow, University of Chicago.
Johnson, Harry M., Student, Cornell University.
Kaye, Alvin M., Senior at Columbia College, Columbia University.
Kelley, Joan M., Vassar College.
Krause, Leonard A., Student, University of Connecticut.
Lathrop, Merle Inez, Instructor, Smith College.
Levin, Norman L., Student, University of Illinois.
Lieb, Rev. Joel R., Graduate Student, St. Louis University.
Lippitt, Ruth H., Student, Wellesley College.
Lyon, John B., Jr., Hamilton College.
MacMillan, Shirley A., Student, Oberlin College.
Mann, David E., Jr., Purdue University.
Maynard, Donald M., Student, Harvard University.
McCashland, Benjamin W., Instructor of Physiology, University of Nebraska.
McDermott, William M., Tufts College.
Newman, Ellen, Radcliffe College.
Rolle, Gloria K., Laboratory Technician, New York University.
Saunders, Richard L., University of Massachusetts.
Scheltema, Rudolf S., George Washington University.
Seale, Joanne K., Pennsylvania College for Women.
Shaw, Edward I., Graduate Assistant in Zoology, University of Missouri.
Stahl, Walter, Massachusetts Institute of Technology.
Tamar, Henry, Graduate Student, New York University.
Tassoni, Joseph P., Assistant in Biology, New York University.
Timm, Rev. Richard W., Graduate Student, Catholic University.
Trefz, Shirley, Temple University.
Urso, Paul, Jr., Student, St. Francis College.
Van Aken, Mary C., Graduate Student, Yale University.
Vernberg, Frank J., Graduate Student, Purdue University.
Vernberg, Winona M., Graduate Student, Purdue University.
Walsh, Winifred, Student, Hunter College.
Weinert, Sanford D., Graduate Student, Harvard University.

4. The Lalor Fellows, 1950

James Danielli, King's College, London, England.
Seymour S. Cohen, University of Pennsylvania.
William P. Jacobs, Princeton University.
Sally M. Kelly, Vassar College.
Alan H. Mehler, University of Chicago.
Stuart Smith, University of Colorado Medical School.

BIOLOGICAL LABORATORY
WOODS HOLE

5. TABULAR VIEW OF ATTENDANCE, 1946–1950

	1946	*1947*	*1948*	*1949*	*1950*
INVESTIGATORS—Total	267	299	326	344	338
Independent	175	182	183	193	198
Under instruction	29	36	42	52	43
Library Readers	38	36	50	55	48
Research Assistants	25	45			49
STUDENTS—Total	122	131	123	128	126
Zoology	57	55	54	55	55
Embryology	30	33	29	31	29
Physiology	26	26	25	27	27
Botany	9	17	15	15	15
TOTAL ATTENDANCE	389	430	449	472	444
Less persons registered as both students and investigators		2	6	2	
	389	*428*	*443*	*470*	*444*
INSTITUTIONS REPRESENTED—Total	*141*	*148*	*158*	*155*	*156*
By Investigators	102	114	117	114	114
By Students	56	56	68	68	67
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	2	1	1	1	2
By Students		1			
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	7	7	8	6	6
By Students	5	3	4	3	2

6. SUBSCRIBING AND COOPERATING INSTITUTIONS, 1950

Subscribing Institutions

Children's Hospital of Philadelphia
Elmira College
Georgetown University Medical School
Hahnemann Medical School
Haskins Laboratories
Indiana University
Marquette University
Morgan State College
National Research Council
Pennsylvania College for Women
Public Health Research Institute of New York City
Radcliffe College
Rutgers University
St. Francis College
Southwestern Medical College
Tulane University School of Medicine
University of Miami
University of New Hampshire
University of Rochester
University of Wisconsin
Vanderbilt University School of Medicine
Wellesley College
Wheaton College
Wilson College

Cooperating Institutions

Amherst College
Argonne National Laboratory
Brooklyn College
Brown University
Bryn Mawr College
College of Mt. St. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Cornell University
Duke University
Emory University
Fordham University
Goucher College
Harvard University
Harvard University Medical School
Hunter College
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly and Company

Madison Foundation
Massachusetts Institute of Technology
Mount Sinai Hospital
National Cancer Institute
New York University College of Medicine
New York University, Heights
New York University, Washington Square College
North Carolina State College
Northwestern University
Oberlin College
Princeton University
Rockefeller Institute for Medical Research
St. Louis University
Sloan-Kettering Institute
State University of Iowa
Temple University
Tufts College Medical School
Union College
University of Chicago
University of Delaware
University of Illinois
University of Kansas
University of Massachusetts
University of Maryland School of Medicine
University of Michigan
University of Minnesota
University of Missouri
University of Oklahoma
University of Pennsylvania Medical School
University of Pennsylvania
University of Vermont Medical School
University of Virginia
Vassar College
Washington University
Wesleyan University
Western Reserve University
Yale University

7. Evening Lectures

The Friday Evening Lectures, 1950

Friday, June 30
Dr. A. E. Szent-Györgyi .... "Life and Muscle."
Friday, July 7
Dr. Arthur W. Pollister .... "Studies in Cytology."
Friday, July 14
Dr. Carroll M. Williams .... "A Hormonal-Enzymatic Mechanism for the Control of Growth and Differentiation in the Cecropia Silkworm."
Friday, July 21
Dr. James Danielli .... "The Phosphatase of Cell Nuclei."
Friday, July 28
Dr. H. H. Plough .... "Radiation Induced Mutations in *Salmonella typhimurium* and Their Genetic Implications."
Friday, August 4
Dr. John Runnström .... "Some Aspects of the Developmental Physiology of the Sea Urchin."
Friday, August 11
Dr. Chester L. Yntema .... "Induction and Differentiation of the Ear."
Friday, August 18
Dr. Robert F. Pitts .... "Renal Control of Acid Base Balance."
Friday, August 25
Dr. Clarence Cottam .... "Wildlife Management in Relation to America's River Development Program."

Other Lectures

Wednesday, August 23
Dr. D. O. Jordan .... "Recent Studies on Desoxyribosenucleic Acid."
Dr. A. G. Ogston .... "Experiments on Hyaluronic Acid."

8. Seminars, 1950

July 5	
Anita Zorzoli	"Alkaline Phosphatase Activity in Normal and Scorbutic Bones."
S. J. Cooperstein	"The Reduction of Janus Green by Isolated Enzyme Systems."
Arnold Lazarow	"The Reduction of Janus Green by Liver Cell Constituents and the Mechanism of Janus Green Staining of Mitochondria."
July 11	
Claude A. Villee	"The Enzymatic Conversion of Ovalbumin to Plakalbumin."
Milton Levy	"Denaturation of Ricin as Affected by pH and Temperature."
Robert C. Warner	"Preparation and Properties of the Conalbumin of Egg White."
July 18	
Charles B. Metz	"Amino Acids and Peptides as Adjuvants in Agglutination of Starfish Sperm by Fertilizin."
Albert Tyler	"Extension of Functional Life Span of Spermatozoa by Amino Acids and Peptides."
J. Runnström	"The Effect of Ca and Jelly Coat Substance on the Agglutination and Respiration of Sea Urchin Spermatozoa."
July 25	
J. W. Green and A. K. Parpart	"The Effect of Metabolic Poisons on Potassium Loss from Rabbit Red Cells."
B. Lucké, R. Ricca and A. K. Parpart	"The Effect of X-rays on Permeability to Water and to Glycols of Arbacia Eggs."
M. H. Jacobs and H. N. Glassman	"Permeability of the Erythrocyte at Different Temperatures."
August 1	
John Tyler Bonner	"Orientation to Heat and Light during Morphogenesis in the Amoeboid Slime Molds."
Donald R. Griffin	"Polarized Light and the Orientation of Bees."
Aurin M. Chase	"On the Nature of Luciferin."
Alan H. Mehler	"Reactions Catalyzed by Isolated Chloroplasts."
August 3	
C. C. Speidel	"Cell Adjustments in Frog Tadpoles Following Implant of Foreign Substances."
Emmanuel Fauré-Fremiet	"Feeding Reactions of Some Predator Ciliates."
Paul A. Nicoll	"Subcutaneous Blood Flow in the Bat's Wing."

August 8

Abraham M. Shanes and Thomas DeKornfeld	"Some Spectrophotometric Observations on Invertebrate Nerve and Its Extracts."
Wolf Vishniac	"Ionic Balance Required by Some Marine Microorganisms and the Effect of Quaternary Ammonium Ions."
Ivor Cornman	"Selective Destruction of Fibroblasts by Desoxycorticosterone in Cultures of Mixed Tissues."
William P. Jacobs	"Polarity of Hormone-Movement as the Indirect Limiting Factor for Seedling Growth."

August 15

Norman Millott	"The Reactions to Shade and the Pigmentation of the Sea Urchin *Diadema antillarum.*"
Harold F. Blum and Margie R. Mathews	"Photorecovery after Ultraviolet Radiation, in Amphibian Larvae."
Alfred F. Bliss	"Action of Alcohol Dehydrogenase on Vitamin A."

August 22

Lillian K. Schneider	"Population Dynamics in *Escherichia coli.*"
Samson R. Gross	"Heterocaryosis Between Opposite Mating Types in *Neurospora crassa.*"
K. C. Atwood	"The Homology Pattern of Induced Lethal Mutations in *Neurospora crassa.*"
Alfred Marshak	"Comparison of the Base Composition of Nucleic Acids of Nuclei and Cytoplasm of Different Mammalian Tissues."

9. Members of the Corporation, 1950

1. Life Members

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.
Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts.
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
Cole, Dr. Leon J., College of Agriculture, Madison, Wisconsin.
Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri.
Jackson, Mr. Chas. C., 24 Congress Street, Boston, Massachusetts.
Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts.
King, Mr. Chas. A.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
MacNaught, Frank M., Woods Hole, Massachusetts.
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts.
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Missouri.
Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pa.

Morgan, Mrs. T. H., Pasadena, California.
Noyes, Miss Eva J.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Scott, Dr. Ernest L., Columbia University, New York City, New York.
Sears, Dr. Henry F., 86 Beacon Street, Boston, Massachusetts.
Shedd, Mr. E. A.
Strong, Dr. O. S., Columbia University, New York City, New York.
Waite, Prof. F. C., 144 Locust Street, Dover, New Hampshire.
Wallace, Louise B., 359 Lytton Avenue, Palo Alto, California.

2. Regular Members

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.
Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.
Albaum, Dr. Harry G., Biology Dept., Brooklyn College, Brooklyn, N. Y.
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota.
Allee, Dr. W. C., 114 Leigh Hall, University of Florida, Gainesville, Florida.
Amberson, Dr. William R., Department of Physiology, University of Maryland, School of Medicine, Baltimore, Md.
Anderson, Dr. Rubert S., Department of Physiology, University of South Dakota, Vermillion, South Dakota.
Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.
Angerer, Prof. C. A., Department of Physiology, Ohio State University, Columbus, Ohio.
Armstrong, Dr. P. B., State College of New York Medical Center at Syracuse, New York.
Atwood, Dr. Kimball C., Department of Zoology, Columbia University, New York 27, New York.
Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.
Baitsell, Dr. George A., Yale University, New Haven, Connecticut.
Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.
Ball, Dr. Eric G., Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.
Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.
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PHYSIOLOGICAL STUDIES OF DIFFERENTIATION IN MYTILUS EDULIS. I. THE OXYGEN UPTAKE OF ISOLATED BLASTOMERES AND POLAR LOBES[1]

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The classic experiments of Wilson (1904), Conklin (1905) and others have demonstrated that the eggs and embryos of many invertebrate animals contain regions of cytoplasm which are determined as to their subsequent fate. A precocious differentiation may even precede cleavage of the eggs, as in some annelids, molluscs, and ascidians. The cytoplasmic regions of these so-called mosiac eggs have been described variously as containing "organ forming" substances or of being chemo-differentiated, but as yet there has been slight progress in defining these mosaic regions in terms of specific physicochemical differences.

Spek (1934) attempted to demonstrate regional cytoplasmic differences by means of vital dyes and concluded on the basis of differential vital staining that a pH gradient existed along the polar axis of various mosaic eggs. Ries (1937, 1939) compared mosaic and regulative eggs by the use of cytochemical techniques. Tests for ascorbic acid, glutathione, benzidine peroxidase, and indophenol oxidase were used, in addition to measurements of rH_2 by means of oxidation-reduction dyes. In some mosaic eggs a localization of these substances or a difference in redox potential could be demonstrated, although the significance of these in the process of differentiation was unclear. Similar investigations on mosaic eggs have been carried out and extended by Reverberi and Pitotti (1940), Pitotti (1947) and Urbani and Mistruzzi (1947). Cytochemical tests have also been used to demonstrate localization of enzymes in mosaic eggs by Lehmann (1948) and Raven (1948).

Although cytochemical techniques have yielded valuable information regarding the chemical heterogeneity of mosaic eggs, they have the disadvantages of being limited in scope as well as being mainly qualitative. Also considerable controversy exists as to the validity of some of the cytochemical techniques (Brachet, 1947). It is apparent that the most desirable approach to this problem would be to carry out direct microchemical analyses and physiological studies of the various cytoplasmic regions. It was with this intention that the present investigation was undertaken.

Owing to the small size of mosaic eggs, it would be difficult to obtain, by cutting the egg into fragments, sufficient amounts of cytoplasm for microchemical studies. It is known, however, that in several mosaic eggs the first cleavage plane normally separates cytoplasmic regions with different presumptive fates. This is particularly striking in the eggs which form polar lobes such as those of Dentalium, Ilyanassa

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and Myzostoma. It seemed that a profitable approach to the problem of cytoplasmic localization would be to take advantage of this natural segregation of cytoplasmic areas and to develop methods for large scale separation of blastomeres in such a form.

The eggs and embryos of *Mytilus edulis* have proven to be particularly suitable for this purpose. Field (1921) has described the early development of *M. edulis* in detail. The first cleavages are similar to those of Dentalium (Wilson, 1904); a polar lobe forms at the vegetal pole of the fertilized egg and is subsequently incorporated into the CD blastomere. Prior to the next cleavage a second polar lobe forms which fuses with the D cell. The first cleavage blastomeres are unequal in size and therefore may be readily recognized.

Investigations on the development of isolated blastomeres and eggs lacking the polar lobes indicate that a typical cytoplasmic localization exists in the Mytilus egg. The polar lobe contains factors which are necessary for the normal development of the larval form and particularly for an apical tuft (to be published).

As a prerequisite for physiological and microchemical studies, it was necessary to develop methods for separating the blastomeres of early cleavage stages of Mytilus. Even on a small scale this is ordinarily difficult, owing to a tough protective membrane surrounding the eggs and an intercellular cement binding the blastomeres together. Fortunately it was discovered that sperm extracts contain lysins which dissolve the enclosing membranes as well as the intercellular cement (Berg, 1950). The use of these lysins permits large scale separation of the blastomeres which subsequently may be used for physiological studies. A method has also been discovered for obtaining large numbers of isolated polar lobes which permits investigations to be carried out directly on the polar lobe itself.

The present report, dealing with the respiration of isolated blastomeres and polar lobes of Mytilus, was undertaken in view of the many attempts in the past to correlate embryonic differentiation with metabolic gradients. Child (1941), for example, has for many years postulated physiological gradients or "respiratory gradients" as controlling factors of differentiation. Ries (1942) also suggests that there is a coupling of oxidation intensity and the oxidase content of regions of mosiac eggs. These conclusions, however, have been based on the results of cytochemical studies, whereas the present techniques permit direct quantitative measurements of the respiratory intensity of different regions of the egg. A preliminary report of this work has recently appeared (Berg and Kutsky, 1950).

METHODS

Mytilus edulis were collected from the San Francisco Bay and stored "dry" in a refrigerator at 4° C. Eggs and sperm were obtained by placing animals in separate bowls of sea water and allowing natural spawning to occur. Although spawning occurs up to one week after storage of the animals at low temperatures, as a rule they were used within the first few days to avoid a progressive deterioration of the eggs.

The procedure for separation of the first cleavage blastomeres is based upon the discovery of lysins in sperm extracts. As previously reported (Berg, 1950), sperm extracts prepared by freezing and thawing of a concentrated sperm suspension contain lysins which dissolve the membrane enclosing the egg and also the intercellular cement binding the blastomeres together. For the present experiments it was convenient to prepare sperm extracts from the testes of *M. californianus* since considerably greater amounts of sperm may be obtained from this species. Several liters of a concentrated sperm suspension were prepared by homogenizing the testes of several dozen males. This was frozen, centrifuged, and the supernatant measured into 10-ml. vials which were stored at −20° C.

Separation of the first cleavage blastomeres was carried out using sperm extract alone or by a combination of sperm extract and calcium-free sea water. With the former method the eggs were fertilized and approximately five minutes before cleavage the excess sea water was pipetted off and 5–10 ml. of sperm extract added. Dissolution of the egg membranes occurred in a few minutes and, as described previously (Berg, 1950), on completion of the first cleavage the blastomeres became partially or completely separated. It was usually necessary to draw the eggs gently in and out of a pipet to increase the percentage of complete separation. Calcium-free sea water also dissolves or prevents the formation of intercellular cement and may be used for blastomere separation. With this method the membranes were removed by means of the sperm extract shortly after fertilization and the eggs were then transferred through several dishes of calcium-free sea water.

The use of sperm extract alone is preferred in some instances since the blastomeres in calcium-free sea water are more fragile and the agitation necessary to ensure complete separation often causes cytolysis. Both methods, however, have been used satisfactorily. After separation, the blastomeres were transferred through several dishes of normal sea water.

Isolated polar lobes were obtained by subjecting membraneless eggs to $MgCl_2$ solutions at the time of polar lobe formation, a procedure which causes a number of the polar lobes to constrict off rather than fuse with the CD cells. Moderate agitation increases the percentage of isolated polar lobes. Since there is considerable variability in the success of this method and the factors involved have not been studied in detail, a fuller report of this method will be published at a later date.

The oxygen uptake of whole eggs, isolated blastomeres, and polar lobes was measured with the Cartesian diver ultramicrorespirometer. Divers were constructed from Pyrex glass according to a design described by Zeuthen (1950), in which the part of the diver containing the biological material is plugged with a hollow glass stopper. Air volumes of the divers ranged from 0.4 μl. to 1.1 μl. and the liquid charge amounted to about two-thirds the size of the air bubble. In paired experiments divers with approximately the same volume were nearly always used since there was a tendency for larger divers to give slightly higher oxygen uptake values. As reported by Zeuthen, these divers are practically gas tight. Blank tests in which pure oxygen or nitrogen was introduced as the bubble resulted in a drift of the equilibrium volume of about 0.1 cm. manometer pressure per hour (after an initial equilibration period).

The flotation vessels were filled with alkaline sodium chloride solution isotonic with sea water. Equilibrium pressures, as determined by raising the diver slightly off the bottom of the flotation chamber, were measured every 10 to 20 minutes depending upon the type of experiment. In nearly all experiments the number of cells introduced into the diver was chosen so as to cause a manometer change of 10–15 cm. per hour.

Immediately after placing a loaded diver in the flotation chamber, the equilibrium pressure decreases rapidly at first and then more slowly. This is probably due to equilibration taking place between the liquid and gas phases of the diver charge. Accordingly actual measurements of oxygen uptake were not begun until after a half hour equilibration period. The error due to a residual drift becomes negligible (less than 0.5 cm. per hour) after about 30 minutes. All experiments were carried out at 20° C.

Oxygen uptake was calculated in the usual manner from the volume of the bubble at equilibrium pressure, the change of the equilibrium pressure per unit time, and the number of cells. Corrections were made for the decrease in oxygen tension in the liquid charge and also for the manometer pressure during the period of measurement.

THE OXYGEN UPTAKE OF ISOLATED BLASTOMERES

AB and CD cells were separated from one another by either of the previously described methods and then transferred through several dishes of sea water. Since AB cells average about 41 μ in diameter and CD cells about 51 μ, they can be distinguished under a low power microscope. The dish from which the blastomeres were to be counted was kept at 10°–15° C. in order to delay cleavage, as it is somewhat easier to distinguish the two types of cells prior to their first cleavage. In each experiment 75 CD cells were selected under the microscope with a braking pipet and introduced into the diver by inserting the tip of the pipet into the diver and slowly expelling the cells so that they settled to the bottom. Loading of the diver was done under a dissecting microscope with the diver held in a fixed position on a small glass plate. One hundred fifty AB cells were selected and loaded similarly into another diver. Air bubbles were measured with a calibrated braking pipet and introduced into the respective divers. The sea water above the air bubbles was then displaced with an alkaline sodium chloride solution, the divers stoppered, placed in the flotation chambers and allowed to equilibrate for approximately 30 minutes.

After loading the divers, the diameters of representative samples of AB and CD cells (usually about 20 to 30 each) were determined with an ocular micrometer. The measured blastomeres were selected from the same batch as that used for the respiration experiment.

The time necessary for counting the cells, loading the divers, etc., caused a delay such that actual oxygen uptake measurements could not begin until nearly three hours after fertilization. At this time the blastomeres had undergone cleavage and were all at least in the two-cell stage. Respiration was measured for an 80-minute period in which time several more cleavages occurred and the embryos consisted of eight or more cells. It might be pointed out here that the cleavage rates of the AB and CD cells are nearly identical during their early development (Fig. 4).

In initial experiments it became apparent that due to biological variability and experimental errors, it would be necessary to carry out a number of experiments and apply a statistical test. To facilitate the statistical analysis paired experiments were carried out, *i.e.*, AB and CD cells were obtained from the same batch of eggs and the oxygen uptake of these determined simultaneously. The data for 12 paired experiments are listed in Table I. Oxygen uptake was first calculated, using the equilibrium volume of the bubble, change in manometer pressure for the period, number of cells, etc., and with the appropriate corrections expressed as μl. O_2 uptake per hour per embryo. Since the amount of cytoplasm in the AB and CD embryo differs considerably, it was decided to express oxygen uptake in terms of unit amount of cytoplasm. The above figures were therefore divided by the average initial volume of the cells for the particular experiment and final comparative values could then be expressed as μl. oxygen uptake/hr./mm.^{3} cytoplasm.

The average of 12 experiments on the respiration of AB embryos, as shown in Table I, is 0.90 ± .032 μl. O_2/hr./mm.^{3} cytoplasm, as compared with 0.78 ± .027 for the corresponding CD embryos. The difference of 13 per cent is significant at the 1 per cent level, and it may be concluded from this that the respiration of CD cytoplasm is significantly lower than that of AB cytoplasm.

TABLE I

Comparison of oxygen uptake of isolated AB and CD embryos for an 80-minute interval three hours after fertilization

Experiment No.	Oxygen uptake of AB embryos 10^{-5} μl. O_2/hr./AB embryo	Average volume of AB cells 10^{-5} mm.^{3}	Oxygen uptake of AB embryos 10^{-1} μl. O_2/hr./mm.^{3} AB cytoplasm	Oxygen uptake of CD embryos 10^{-5} μl. O_2/hr./CD embryo	Average volume of CD cells 10^{-5} mm.^{3}	Oxygen uptake of CD embryos 10^{-1} μl. O_2/hr./mm.^{3} CD cytoplasm
1	3.1	3.95	7.8	4.8	7.54	6.4
3	2.9	3.27	8.9	4.9	6.62	7.4
4	3.0	3.46	8.7	5.2	7.10	7.3
6	3.2	3.40	9.4	5.5	6.62	8.3
8	3.5	4.24	8.3	6.7	8.52	7.9
9	3.0	3.82	7.9	5.4	7.62	7.1
10	3.0	3.58	8.4	4.8	7.35	6.5
11	3.5	3.66*	9.6	6.9	7.35*	9.4
12	2.8	3.66*	7.7	5.8	7.35*	7.9
13	3.8	3.95	9.6	6.0	7.54	8.0
14	3.5	3.52	9.9	5.9	7.10	8.3
15	4.0	3.46	11.5	6.3	6.80	9.3
	Average		9.0 ± .32 S.E.			7.8 ± .27 S.E. (13% lower than respiratory rate of AB embryos). t = 5.6, sig. at 1% level

* Diameters not measured. Average of all experiments used.

In addition to the above experiments three others were carried out which were not paired, *i.e.*, the AB and CD cells did not come from eggs of the same female. If these are included with the data of Table I, the rate of O_2 uptake of the CD embryos is found to be 15 per cent less than that of AB embryos. Statistical treatment of this unpaired series indicates a difference significant at the 1 per cent level.

THE OXYGEN UPTAKE OF ISOLATED POLAR LOBES

The polar lobe is incorporated into the CD cell at the close of the first cleavage and one might reasonably suspect that any physiological differences existing between the first cleavage blastomeres might be due to the polar lobe. Thus, the lower respiratory rate of the CD cells might be owing to the presence of the polar lobe material.

Polar lobes were isolated by the use of sperm extracts and $MgCl_2$ solutions, as described previously, and transferred through several dishes of normal sea water. In nine experiments 425–475 lobes were drawn up into a fine braking pipet and loaded into a diver in the same manner as described for AB and CD cells. A sample (30 to 40) of polar lobes was removed from the same dish and their diameters measured. The average diameter of isolated polar lobes is almost 30 microns which is about 10 microns less than the AB cells. AB cells were also present in the dish with isolated polar lobes and it was sometimes difficult to distinguish between the largest of the polar lobes and the smallest of the AB cells, particularly when several hundred polar lobes had to be selected rather rapidly. In almost all experiments a few AB cells were thus unavoidably included in the diver with the polar lobes. These served a useful purpose, however, since the presence of swimming AB embryos in the diver after the respiration measurements served as an indication of the validity of the test. In calculating the respiration of the polar lobes a correction was made for the respiration of these AB cells.

TABLE II

Comparison of oxygen uptake of isolated polar lobes and whole eggs

Oxygen uptake of whole eggs during time of polar lobe formation 10^{-5} μl./hr./egg	Average volume of eggs 10^{-5} mm.^{3}	Oxygen uptake of eggs during time of polar lobe formation 10^{-1} μl. O_2/hr./mm.^{3} egg cytoplasm	Oxygen uptake of isolated polar lobes 10^{-6} μl./hr./polar lobe	Average volume of polar lobes 10^{-5} mm.^{3}	Oxygen uptake of polar lobes 10^{-1} μl. O_2/hr./mm.^{3} polar lobe cytoplasm
6.7*	12.3	5.4	5.6	1.39	4.0
6.8*	13.7	5.0	6.8	1.71	4.0
6.9*	12.6	5.5	4.8	1.56‡	3.1
6.6	12.5	5.3	8.7	1.84	4.7
7.3	12.4	5.9	6.7	1.50	4.5
7.5	12.0	6.3	5.2	1.22	4.3
6.8	12.2‡	5.6	6.3	1.56‡	4.0
6.8	11.8	5.8	4.9	1.39	3.5
6.3	12.2‡	5.2	8.1	1.41	5.7
Average		5.6 ± .13 S.E.			4.2 ± .25 S.E. (25% lower than respiratory rate of whole egg cytoplasm. t = 4.9, sig. at 1% level)

* Average of two or more experiments on the same batch of eggs.

‡ Diameters not determined. Average of all experiments used.

Actual oxygen consumption measurements were carried out for a period of at least three hours. The polar lobes were examined afterwards, and a few tests were discarded because of a marked clumping of the lobes. This, coupled with the absence of swimming AB embryos, suggested that alkali had crept past the air bubble and mixed with the sea water.

For purposes of comparison the respiration of whole eggs was determined during the time of polar lobe formation. A period of 20 minutes was chosen, beginning 60 minutes after fertilization which is approximately the time at which the polar bulge first appears. Fertilized eggs were first treated to remove the membranes and then washed with sea water. In each experiment 50 to 75 of these were loaded into a diver as rapidly as possible, so as to allow sufficient time for the diver to equilibrate. The diameters of representative eggs were then determined during the equilibration period.

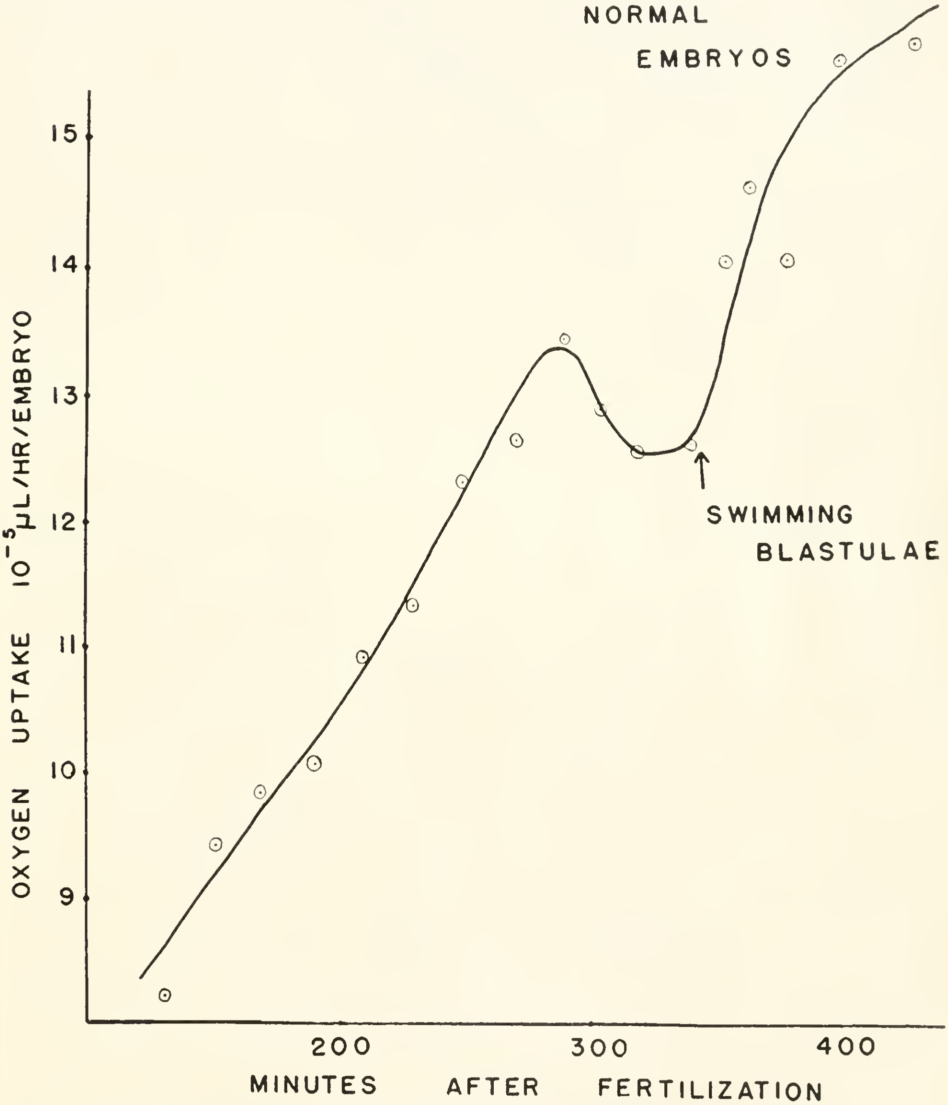

FIGURE 1. Oxygen consumption rate of normal *Mytilus edulis* embryos. 250 embryos in Cartesian diver.

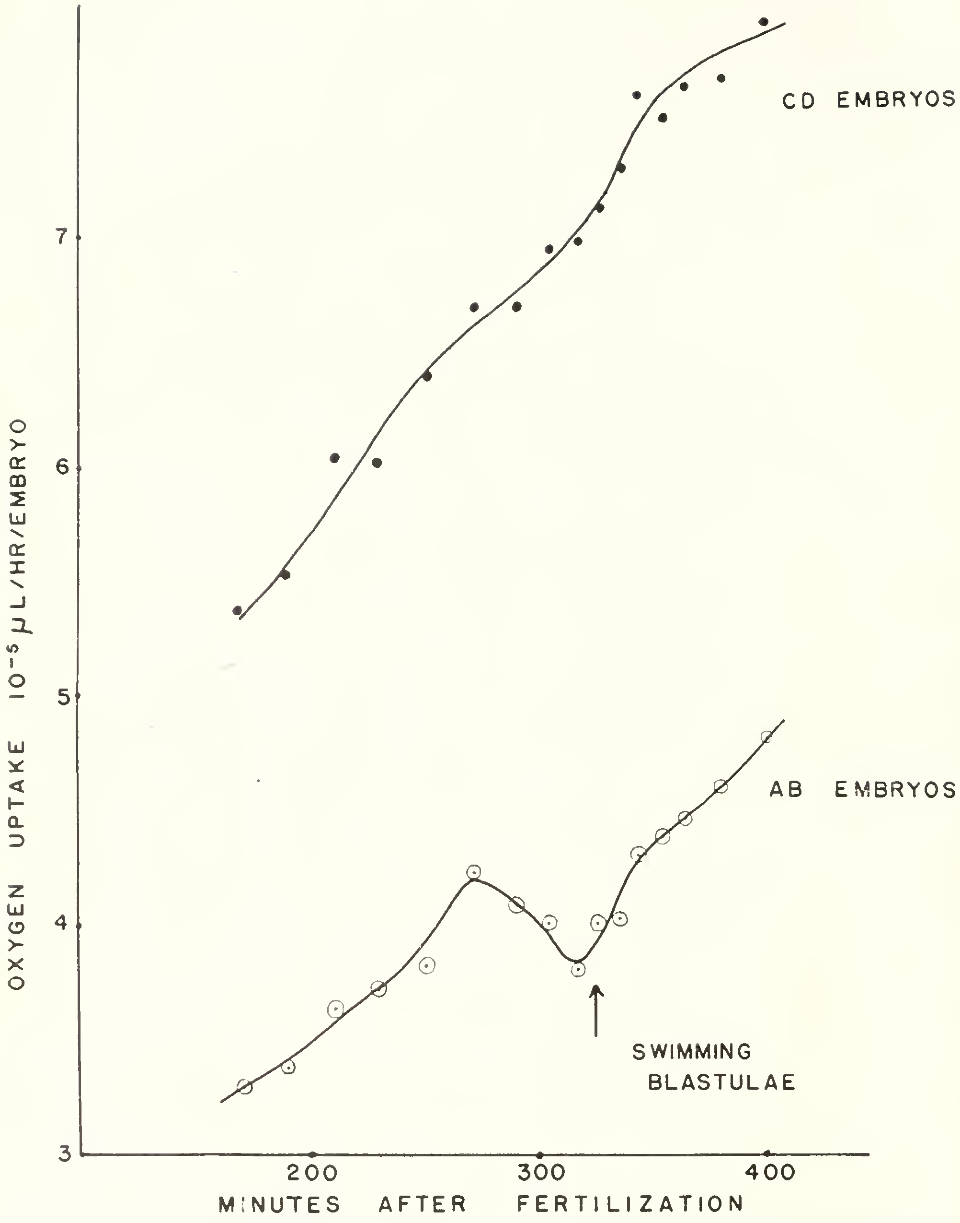

FIGURE 2. Oxygen consumption rate of AB and CD embryos. Average of seven experiments, each with 75 CD or 150 AB embryos in diver.

The data for the oxygen uptake of whole eggs and isolated polar lobes are summarized in Table II. Again, for the purpose of comparison, the oxygen uptake is expressed in terms of oxygen consumed per unit amount of cytoplasm. The average oxygen uptake of whole eggs during the time of polar lobe formation is

0.56 ± 0.013 μl. O_2/hr./mm.^{3} egg cytoplasm as compared to 0.42 ± 0.025 for the polar cytoplasm. The difference of 25 per cent is significant at the 1 per cent level (data treated as an unpaired series). It thus appears that the lower respiration of the CD cells can be explained, at least in part, on the basis of a lower oxygen consumption of the polar lobe cytoplasm.

Rate of Oxygen Uptake During Early Development

In a few initial experiments the rate of oxygen uptake of normal embryos was determined for a period of 7 or 8 hours after fertilization. As would be expected, the respiratory rate of the embryos gradually rises as development proceeds, as has been shown to be the general rule in embryonic development. It was discovered, however, that shortly before the embryos hatched from the membranes and began swimming, there was a decrease in the respiratory rate. After hatching of the blastulae the rate of oxygen consumption increased sharply and then more slowly. This phenomenon was investigated more thoroughly, and a typical oxygen uptake curve is shown in Figure 1. This curve was plotted from the data of a single experiment in which 250 fertilized eggs had been placed in the diver, and it illustrates this interesting decrease in rate of oxygen uptake preceding swimming. There was some variation in the extent of the decrease in different batches of eggs; in the particular curve figured, the decrease is somewhat more pronounced than in other determinations.

Some of the experiments on the respiration of isolated blastomeres were carried out for as long as six hours after fertilization. In these, there seemed to be a tendency for the oxygen uptake of AB embryos to decrease toward the end of the experiment. This was also investigated more thoroughly, and, due to a considerable scattering of points of the respiration curves, it was necessary to average a number of experiments together. Figure 2 illustrates the average of 7 experiments each (a total of 525 CD cells and 1050 AB cells) of the respiration of isolated blastomeres

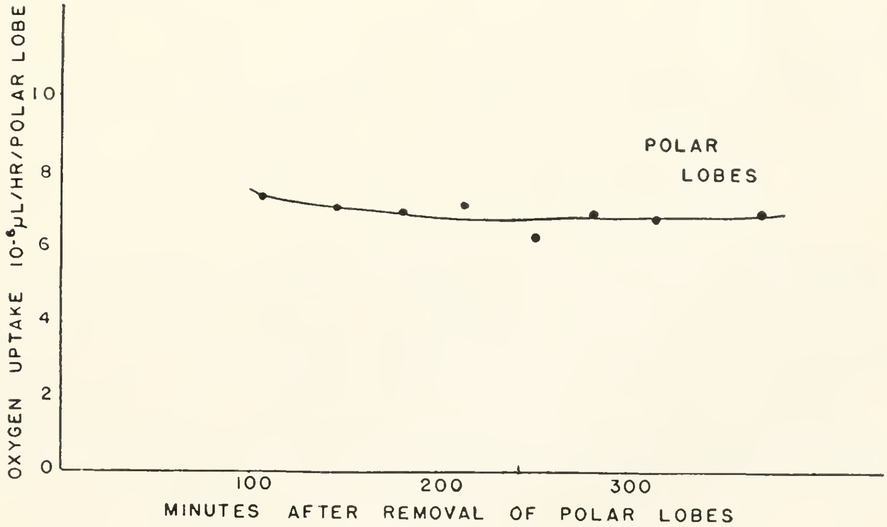

Figure 3. Oxygen consumption rate of isolated polar lobes. 467 polar lobes in diver.

up to 7 hours after fertilization. From this, it is clear that the decrease in respiration of whole embryos is due to a decrease in respiration of the derivatives of the AB cells. Whether the oxygen uptake curve of the CD embryos is a straight line or perhaps exhibits a slight decrease prior to hatching is open to question. It may be that these variations in slope are merely due to experimental errors.

It seemed possible that the above difference in respiratory rates of AB and CD embryos might be related to differential rates of cell division. Accordingly, counts were made of the number of cells composing AB and CD embryos at various stages of development (Fig. 4). It is evident that cell division in AB embryos decreases in rate and ceases at about the time the rate of oxygen consumption drops, whereas cell division continues unabated in CD embryos. Thus, there seems to be some correlation between rate of cell division and the respiratory rate of the AB embryos. The gradual rise in oxygen consumption after hatching, despite arrest of cell division, may be due, in part, to the increasing swimming activity of the embryos. The existence of a relation between oxygen consumption and mitosis has long been recognized, and in general, the more rapid the cleavage, the greater the increase in oxidative metabolism. Why, in this particular instance, there should be a decrease in oxygen consumption with cessation of cell division is not clear. It may be that there is a time lag in the relationship and that there is an overshooting, so to speak, of the rate of oxygen consumption even after cell division has ceased.

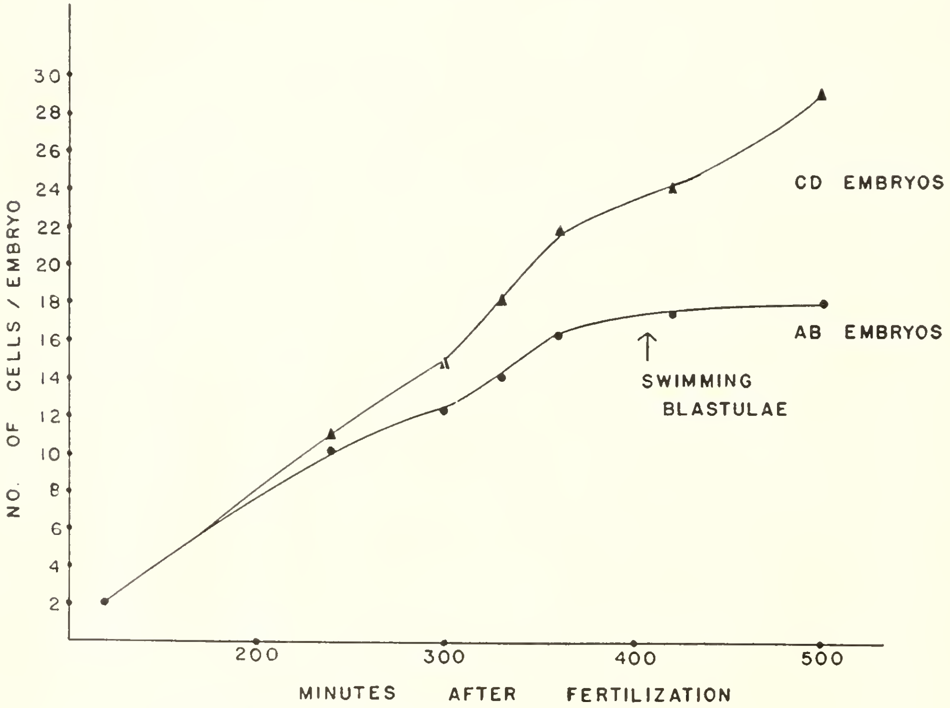

FIGURE 4. Cleavage rates of AB and CD embryos at 18° C.

The oxygen uptake rate of isolated polar lobes was followed for a period of three to four hours after their constriction from the eggs. A single experiment in which 467 polar lobes were placed in the diver is shown in Figure 3. It is significant that the polar lobe cytoplasm, at least when isolated, does not participate in the general rise in respiratory rate as exhibited by the whole eggs or isolated blastomeres. Although only a single curve is presented, five other experiments also indicated that there is no change in the respiratory rate of the isolated polar lobes.

The fact that polar lobes do not show the gradual increase in oxygen consumption indicates that the increase itself is coupled with nuclear and cytoplasmic divisions rather than being an inherent characteristic of the cytoplasm itself. Although the isolated polar lobes undergo cyclic phases of activity (ameboid activity and forming what appears to be a miniature polar lobe), no changes in oxygen consumption were detected during these periods.

Discussion

The possibility of quantitative differences in the respiration of various regions of eggs or embryos has long been a subject of speculation. Thus, in the present experiments wherein such differences have been demonstrated, it is of importance to analyze the possible sources of errors. Previous to the oxygen uptake measurements, the AB and CD cells were subjected to identical treatment, and it is unlikely that any external factors were operating to change their physiological characteristics. There is no reason to believe that a short treatment of the eggs with sperm extract or calcium-free sea water has any immediate or permanent effect on the rate of respiration.

There are, of course, errors which may be of considerable magnitude in the measurement of respiration with the Cartesian diver. Each diver has a characteristic drift of the equilibrium pressure which depends upon the size of the air volume, temperature at which it is loaded, etc. This drift is considerable at the beginning of an experiment and amounts to as much as 50 per cent of the respiration of the cells. Within about 30 minutes, however, it decreases to 1 to 5 per cent of the respiration. In order to avoid the initial large error due to drift, measurements were as a rule not begun until after a thirty-minute equilibration period. Even after this period there is a slight drift of the divers; however, this tends to cancel out in the comparative experiments since the drift is in the same direction for all divers. To avoid other possible sources of errors, the divers used for measuring the respiration of the isolated blastomeres were matched as much as possible in general dimensions and in air volume. In addition, they were alternated, the diver used for AB cells in one experiment being loaded with CD cells in the next.

In order to compare the oxygen uptake of embryos of different sizes, it was necessary to express the respiration in terms of unit amount of cytoplasm as calculated from the initial diameters of the cells. The diameters of the cells were measured with an accuracy of one or two microns and the average of 20 or more measurements used in each experiment. While there is an error in this average, it is a random error and with the statistical test used it would not influence the significance of the results. A possible source of systematic error in these measurements is a differential flattening of the cells during the time of measurement; however, there is no evidence that this occurs.

The respiratory rate of whole eggs or isolated blastomeres may vary as much as 20 per cent, as indicated in Tables I and II. While it is difficult to distinguish errors of technique from biological variability, the results of duplicate tests suggest that this is mainly due to the latter. Variations of oxygen uptake of considerable magnitude have been observed, for example, in sea urchin eggs (Krahl, 1950).

In the experiments on the respiration of polar lobes, it was suspected that pretreatment with $MgCl_2$ may have caused a decrease in rate of oxygen uptake. This cannot be tested directly since there is no other known method for obtaining polar lobe cytoplasm in sufficient quantity for respiration measurements. However, as an indirect test, a series of experiments were made on whole eggs which had been exposed to $MgCl_2$ solutions much in the same way as for removing the polar lobes. The average oxygen uptake of the treated eggs was not significantly different from the controls. There still remains the rather unlikely possibility that $MgCl_2$ has a differential effect on the respiration of polar lobes as compared to the remaining cytoplasm; however, at present there is no method for conveniently testing this. The fact that the CD cells, which contain the polar lobe cytoplasm, show a low oxygen uptake would tend to confirm the conclusion that the low respiration of the polar lobe cytoplasm is not artificially induced. This also indicates that the lower respiration of the polar lobes is not due to the absence of nuclear material.

In Table II the oxygen uptake of polar lobes was compared with that of whole eggs. The latter, however, contain the lower respiring polar lobe material and for a comparison between the polar lobe cytoplasm and the remaining cytoplasm, one should include a correction for this. If one adjusts for the amount of polar lobe material actually pinched off (which has a volume of about 11 per cent of that of whole eggs) the correction is negligible. If it is assumed that the volume differences between AB and CD cells are due to polar lobe material, and furthermore that all polar lobe cytoplasm respires at the same low rate, then the correction becomes considerable. Such a calculation indicates that polar lobe cytoplasm has an oxygen consumption per unit volume 35 per cent lower than that of the remaining cytoplasm.

The assumptions made in the above calculation would be difficult to check; however, another calculation lends some support to the above interpretation. The respiration of the polar lobes cannot be compared directly with the AB cytoplasm, since by the time the AB cells can be counted and loaded in the diver, they have continued development and their rate of oxygen uptake increased accordingly. It is possible, however, to extrapolate the oxygen consumption curves of AB embryos (such as in Fig. 2) back to the time of completion of the first cleavage. If these values are expressed in terms of unit amount of cytoplasm and compared with the polar lobes (from Table II), the polar lobes are found to have an oxygen consumption 44 per cent lower than that of AB cytoplasm. While the above calculations are very approximate, they do, however, indicate that the value of 25 per cent obtained from the data of Table II is too low, and that there is probably a greater differential in rate of oxygen consumption between the polar lobe and the remaining cytoplasm than appears on first inspection.

Since the volume of an isolated polar lobe is about one fifth that of a CD cell, the lower respiratory rate of the isolated polar lobe cannot by itself account for the difference in respiratory rates of AB and CD cells. There is reason to believe, however, that not all the polar lobe cytoplasm is isolated by the $MgCl_2$ treatment.

Visual inspection of normally cleaving eggs indicates that the total polar lobe material is appreciably greater than the amount removed. If it is assumed that this remaining material has an equally low respiration as the isolates, then the difference between AB and CD cells can be reasonably accounted for.

Gradients of various types have been suggested as being important factors in embryonic development. The nature of these has not always been made clear; however, several investigators have suggested that they are physiological, or more specifically, metabolic in nature. Child (1941), for example, has proposed on the basis of differential reduction of dyes, a metabolic gradient along the polar axis as a controlling factor in development, particularly of the sea urchin. This has been suggested and often been interpreted as a gradient of oxidative activity. Considerable controversy has existed with regard to this interpretation, and at present there is a tendency to question it, especially in view of the fact that Holter and Lindahl (1940) found no difference in oxygen uptake of animal and vegetal halves of sea urchin embryos.

Although the present data show a regional difference in oxidative metabolism of Mytilus eggs, it cannot be concluded that this difference exists in the form of a gradient. Also, whether this difference is directly coupled with the differentiation of these areas is a point which must await further investigation.

The results of Navez and Harvey (1935), Shapiro (1935), and Ballentine (1940) indicate that oxidative enzymes are attached to submicroscopic granules which can be shifted by centrifugation. The polar lobe cytoplasm is relatively free of granules of the larger visible size and may be lacking to some extent in the smaller ones with attached oxidative enzymes. While there is no proof for this, it is a possible explanation for the lower oxidative rate of the polar lobe cytoplasm.

Ries (1939) has shown by cytochemical methods that specific regions of mosaic eggs are particularly abundant in oxidative enzymes. A striking example of localization of oxidases occurs in the myoplasm of the ascidian egg and one might suppose that this area would have a higher oxidative metabolism. Holter and Zeuthen (1944), however, found no difference between the oxygen uptake of the anterior and posterior blastomeres of the four-cell stages of Ciona.

The present work is the first indication that regions of a mosaic egg may be characterized by different respiratory intensities; however, it might be pointed out that the results are not particularly in accordance with those of cytochemical studies. Pitotti (1947) has suggested that oxidative enzymes tend to be correlated with cytoplasmic areas, the developmental fates of which are to form organs of movement. Thus, the ectoplasm of Beröe eggs, the myoplasm of the ascidian egg, and the polar lobe of Myzostoma are positive with cytochemical tests for oxidative enzymes. While it is not known if the polar lobes of Mytilus exhibit a localization of oxidative enzymes as revealed by cytochemical tests, it is of interest that the respiratory intensity of the polar lobe is lower rather than higher than that of the remaining cytoplasm.

SUMMARY

1. The oxygen consumption of isolated blastomeres and isolated polar lobes of the eggs of *Mytilus edulis* was measured by means of the Cartesian diver microrespirometer. The average oxygen uptake of CD embryos is $0.78 \pm .027$ μl. O_2/hr./mm.^{3} CD cytoplasm. This is 13 per cent lower than the average oxygen

uptake of AB embryos (0.90 ± 0.032) over a comparable period of development.

2. This lower respiratory rate of CD cytoplasm is undoubtedly due to the presence of the polar lobe cytoplasm. Isolated polar lobes have an oxygen uptake of 0.42 ± .025 μl. O_2/hr./mm.^{3} cytoplasm, which is 25 per cent lower than the average oxygen uptake of whole eggs during the time of polar lobe formation (0.56 ± .013). Calculations by several methods suggest that the difference in respiratory rate between the polar lobe and the remaining egg cytoplasm may be appreciably greater than the above figure.

3. The rate of oxygen consumption of polar lobes remains constant after isolation from whole eggs whereas those of AB and CD embryos, as well as whole embryos, exhibit a gradual rise during subsequent development. Prior to hatching, whole embryos show a temporary decrease in oxygen consumption. This has been found to be due to a decrease in oxygen uptake of derivatives of the AB cell. Isolated CD embryos exhibit a steady rise in respiratory intensity during this period. This difference in rate of oxygen consumption of AB and CD embryos prior to hatching can, to some extent, be correlated with their rate of cell division.

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THE EFFECTS OF CERTAIN ANTITHYROID DRUGS ON THE UPTAKE OF RADIOACTIVE IODINE BY THE FROG THYROID[1]

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Within recent years a number of chemical substances which inhibit the activity of the thyroid gland have been extensively studied by Astwood (1943), MacKenzie and MacKenzie (1943), Astwood, Bissell and Hughes (1945), McGinty and Bywater (1945), VanderLaan and Bissell (1946a). In general, these antithyroid substances fall into three classes: (1) thiourea and its derivatives, (2) aniline derivatives, including the sulfonamides and other aminobenzene compounds, (3) thiocyanates.

One method of approach to the problem is the use of radioactive iodine (I^{131}) as a tracer for following the course of injected iodine in animals treated with thyroid-inhibitors. With radioactive iodine tracer techniques, it has been demonstrated by Franklin, Lerner and Chaikoff (1944) and Rawson, Tannheimer and Peacock (1944) that the oral administration of thiouracil to rats and chicks for periods of several days results in a marked reduction in the natural capacity of thyroid tissue to concentrate iodine. In another study, Larson, Keating, Peacock and Rawson (1945) demonstrated that a single injection of 10 mg. of thiouracil markedly inhibited the collection of radioactive iodine by the chick thyroid.

Such studies, for the most part, have dealt with warm-blooded animals. The present paper is a report of experiments carried out on the frog to determine (1) whether frog thyroids treated with thyroid-inhibitors concentrate iodine as effectively as those of control animals; (2) whether there are differences in the action of the three groups of antithyroid compounds; (3) whether there are any significant differences between warm-blooded and cold-blooded forms with respect to the mechanisms involved in the goitrogenic effects of these substances.

MATERIALS AND METHODS

Male and female frogs (*Rana pipiens, Rana clamitans* and *Rana palustris*) of 50 to 65 grams body weight were kept in a large screened cage under dripping water in an air-conditioned room at 5° C.

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In the first series of experiments, groups of experimental animals were given single sub-cutaneous injections of the drugs to be tested. Those receiving thiourea or sulfanilamide were given 0.5 cc. of a 10 per cent solution; those receiving potassium thiocyanate were given 1 cc. of a 0.2 per cent solution. Twenty-four hours after the drug injection a dose of approximately 22.5 microcuries of carrier-free radioactive iodine in a sodium bisulphite solution was injected intraperitoneally into both experimental and control animals. Uniform groups of animals were sacrificed 20 minutes, 40 minutes, one hour, three hours, 8 hours, 24 hours, and 48 hours after injection of the radioactive iodine. The thyroids were removed, rinsed in frog Ringer's solution, blotted, weighed, pressed flat on clean microscope slides, and radioactivity counts made.

In the second series of experiments the experimental animals were given the same doses of the test compounds as above. At intervals of one hour, three hours, 8 hours, 24 hours, 48 hours, and, in some cases, 72 hours after injection of the test compounds, groups of animals were injected intraperitoneally with 22.5 microcuries of carrier-free radioactive iodine in a solution of sodium bisulphite. The control animals received only the radioactive iodine. The animals were sacrificed three hours after the injection of labeled iodine. The thyroids were removed and prepared for reading as above.

The first and second series of experiments showed that potassium thiocyanate disappeared from the circulation approximately 24 hours after its injection into the animal. For this drug a third series of experiments was performed in order to see if the curve for its effectiveness at successive time intervals was similar to the curves for thiourea and sulfanilamide. In this series of experiments the drug was injected and at one minute, 20 minute, 40 minute, one hour, three hour, 8 hour, and 12 hour intervals, the radioactive iodine was injected into experimentals and controls; all animals were killed three hours after injection of the iodine.

In the three series of experiments the collection of radioactive iodine by the thyroid glands was detected by serial counting at two-minute intervals with a Geiger-Muller counter. In each group of animals the count of radioactive iodine collected by the thyroids of the experimental animals was compared with that collected by the thyroids of the untreated controls and expressed as per cent of the control uptake of radioactive iodine. The count of radioactive iodine collected by the thyroids of the experimental groups divided by that of the control animals determines this per cent of control uptake.

RESULTS

The thyroid glands of the control animals were normally pale and to the naked eye did not appear enlarged. The weights of these glands varied between 0.0008 and 0.0014 gm. and averaged 0.001 gm. Animals treated with thiourea had thyroid glands which were dark red, obviously vascular and enlarged. The weights of the thyroid glands of these animals varied between 0.0014 and 0.0023 gm. and averaged 0.0019 gm. Animals treated with sulfanilamide had thyroid glands which were dark red, vascular and much enlarged. The weights of this group of thyroids averaged 0.0015 gm. and varied between 0.0012 and 0.0018 gm. The thyroid glands of the potassium thiocyanate-treated animals were dark red, quite vascular and enlarged. The weights of the thyroid glands of these animals averaged 0.0012 gm. and varied between 0.0010 and 0.0014 gm. The injection of the drugs into the experimental animals produced thyroid glands larger than those of the control animals in every instance. The increase in volume of the glands was apparent to the naked eye and seems to have been greater than was the increase in weight.

Effects of thiourea and sulfanilamide

The effect of a single injection of 0.5 cc. of a 10 per cent solution of thiourea upon the uptake of radioactive iodine by the frog thyroid is shown graphically in Figure 1. It will be seen that the curve for the experimental animals follows that

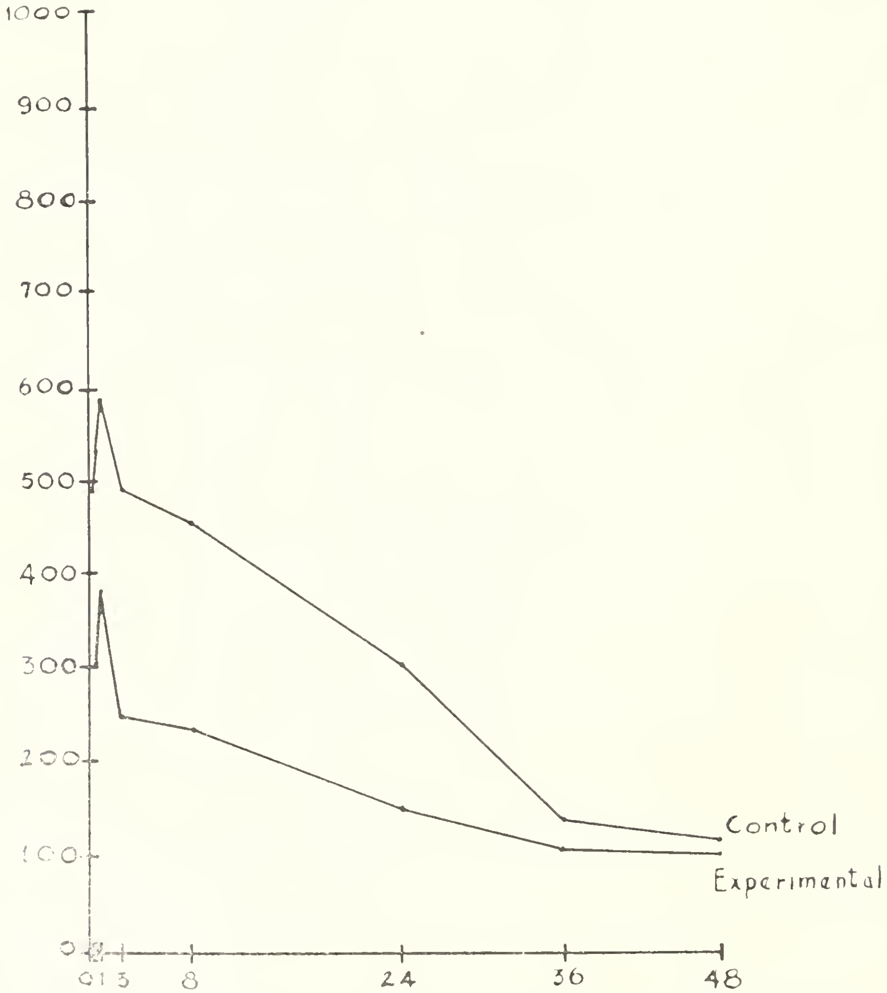

FIGURE 1. The effectiveness of thiourea as an inhibitor of the uptake of I^{131} by the frog thyroid at successive time intervals after injection of the drug. Each point on the graph represents the average of two separate series of experiments. The ordinate shows radioactivity counts per two minutes and the abscissa indicates the time intervals in hours after injection of radioactive iodine.

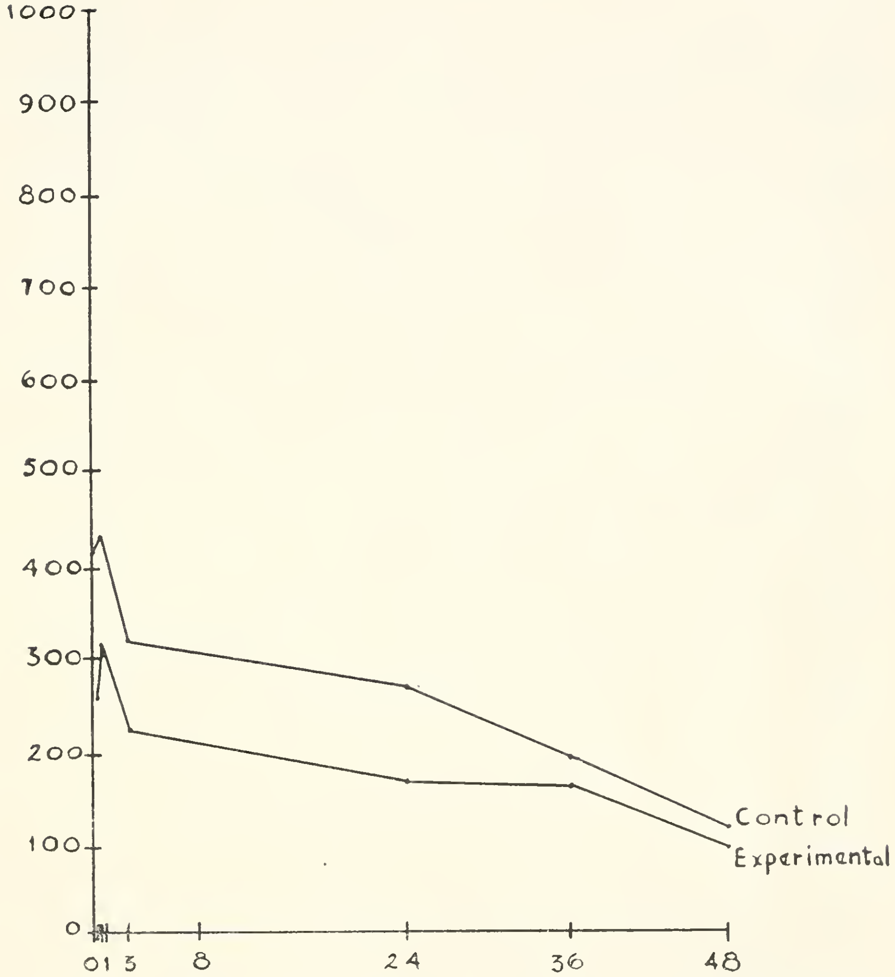

FIGURE 2. The effectiveness of sulfanilamide as an inhibitor of the uptake of I^{131} by the frog thyroid at successive time intervals after injection of the drug. Each point on the graph represents the average of two separate series of experiments. The ordinate shows radioactivity counts per two-minute intervals, and the abscissa indicates the time intervals in hours after injection of radioactive iodine.

for the controls but is consistently lower. The thiourea-treated animals show an uptake which is 62.0 per cent of that of the controls 20 minutes after the injection of radioactive iodine (24 hours and 20 minutes after administration of thiourea). The percentage falls steadily to a level of 48.5 per cent at the 24 hour period (48 hours after thiourea administration). Then, as would be expected, a gradual recovery is exhibited so that by 72 hours after thiourea administration the iodine uptake of the treated thyroids is approaching that of the controls. It is clearly indicated, therefore, that thiourea does affect the thyroid's ability to take up iodine and that,

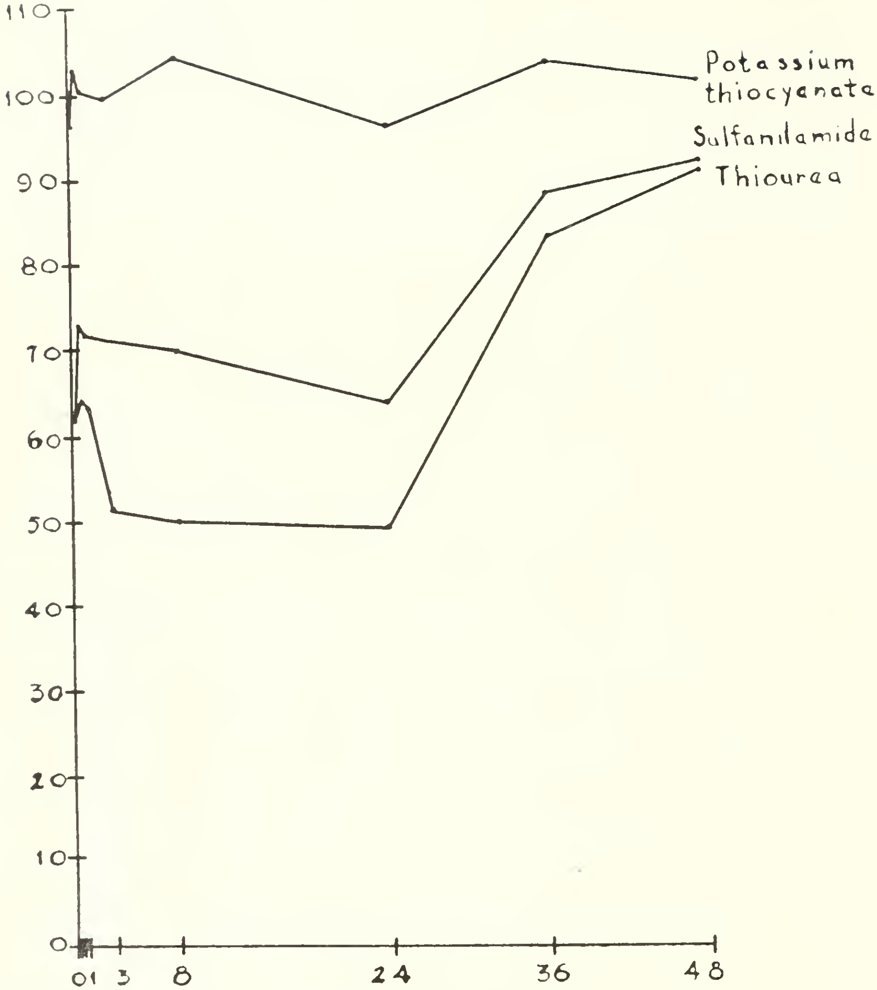

FIGURE 3. The per cent of control uptake of I^{131} by the frog thyroid is compared for thiourea-, sulfanilamide- and potassium thiocyanate-treated animals. Each point on the graph represents the average of two separate series of experiments. The ordinate shows the per cent of the control uptake of I^{131}, and the abscissa indicates the time interval in hours after its injection.

in the frog, a single dose of 0.5 cc. of a 10 per cent solution of thiourea has a maximum effectiveness about 48 hours after the drug is given.

The data for sulfanilamide are graphically presented in Figure 2. The results are clearly similar to those obtained by thiourea treatment, but the effect (at this dosage level) is somewhat less marked, the maximum reduction being only to 63.7 per cent of that of the controls. Another basis for comparison is given in Figure 3 where the curves are based on percentages of control uptake.

After the experiment in which the radioactive iodine was given 24 hours after administration of the thyroid-inhibiting drug and its uptake followed over a 48-hour

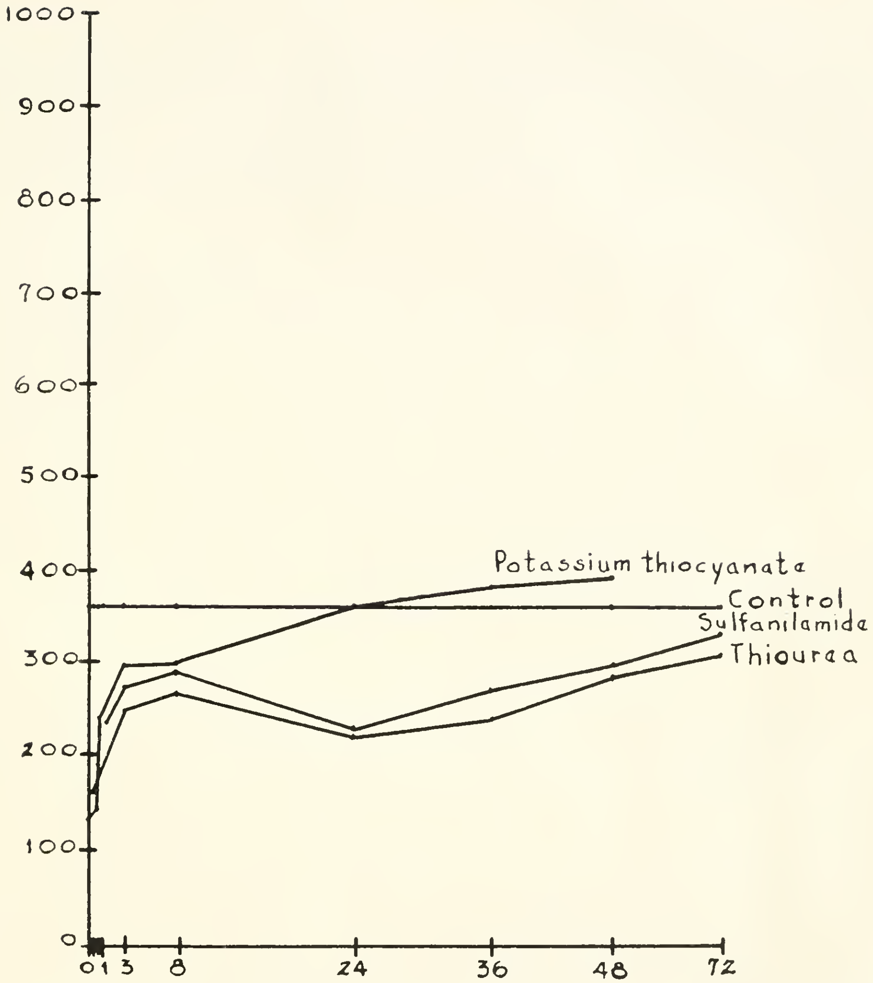

FIGURE 4. The rate of radioactive iodine absorption by frog thyroids treated with thiourea, sulfanilamide and potassium thiocyanate. Each point on the graph represents the average of two separate series of experiments for each drug. The ordinate shows the radioactivity counts per two-minute interval, and the abscissa indicates the time intervals in hours after injection of radioactive iodine.

period, a series of experiments was undertaken in which the radioactive iodine was given at varying times after the drug. The data for thiourea show that the drug effectively blocks iodine absorption almost immediately after its injection. In fact, the maximum effectiveness is seen in the animals which were injected with radioactive iodine only one hour after thiourea injection. With longer intervals the effect is gradually reduced although it is still perceptible after three days. For sulfanilamide the results are similar although again the degree of effectiveness with this dosage is somewhat less than that shown by thiourea.

Effects of potassium thiocyanate

The results of the first series of experiments with potassium thiocyanate showed no significant reduction in iodine uptake by the thyroids of the experimental animals as compared with the uptake by the thyroids of the control animals. The explanation of the failure became apparent, however, when the second series of experiments was carried out. The results of these experiments show that potassium thiocyanate is very effective as an agent for inhibiting iodine uptake by the thyroid, but is rapidly removed from the circulation.

In order to ascertain whether the curve for the effectiveness of potassium thiocyanate at successive time intervals after its injection is similar to the curves for thiourea and sulfanilamide, a third series of experiments was performed in which radioactive iodine was given at intervals from one minute to 48 hours after thiocyanate administration. The results of this study show that the form of the curve for potassium thiocyanate is essentially the same as that for thiourea and sulfanilamide but is displaced to the right; that is, the maximum effect is exerted earlier and the effectiveness declines more quickly. It is to be noted that the uptake of iodine in these thiocyanate-treated animals ultimately reached a level higher than that seen in untreated controls (Fig. 4). This may be due to the increase in number of cells in the hypertrophied glands caused by the initial goitrogenic effect and the subsequent removal of the block to iodine uptake resulting from the rapid decline in effectiveness of the potassium thiocyanate.

Discussion

In the present investigation, the uptake of radioactive iodine by the frog thyroid was ascertained after the injection of three antithyroid drugs (thiourea, sulfanilamide and potassium thiocyanate). From the foregoing results it is clear that thiourea, sulfanilamide and potassium thiocyanate, when injected into normal frogs in doses that are not in the usual sense to be considered toxic, induce a pronounced enlargement of the thyroid gland. These results are similar to those observed by Astwood, Sullivan, Bissell and Tyslowitz (1943) with thiourea and certain sulfonamides on the rat thyroid. Astwood (1943), VanderLaan and Bissell (1946b), and Wolff, Chaikoff, Taurog and Rubin (1946) obtained the same results with potassium thiocyanate. These drugs act primarily by blocking the utilization of iodine, and thyroid hyperplasia and hyperemia occur as secondary compensatory responses to the increased thyrotropic hormone output by the pituitary. The basic system governing the structure and function of the thyroid gland appears to be an interrelation and balance between the thyrotropic activity of the anterior pituitary and the production of a calorigenic substance from the thyroid gland.

By the use of the radioactive iodine technique, it was found that thiourea and sulfanilamide still exert a pronounced inhibitory effect on the thyroid twenty-four hours after injection, but this prolonged effect was not obtained with potassium thiocyanate. Collection of radioactive iodine by thyroids of potassium thiocyanate-treated frogs is similar to that observed for the rat thyroid by Franklin, Chaikoff and Lerner (1944). They observed that thyroid slices *in vitro* fail to absorb radioactive iodine in the presence of potassium thiocyanate. However, the thyroids of some potassium thiocyanate-treated frogs concentrated a greater per cent of the injected labeled iodine than did their controls, but this effect was observed only when the time interval after the injection of the drug was prolonged to the point at which its disappearance from the circulation had occurred. The results of the present investigation show that potassium thiocyanate is rapidly removed from the circulation, but so long as a high concentration is present in the blood, the uptake of radioactive iodine by the frog·thyroid is definitely depressed. The inhibitory action of this drug was observed after a single injection of a 0.2 per cent solution of potassium thiocyanate, provided the labeled iodine is injected and the thyroids removed at a time when potassium thiocyanate is still present in the circulation.

The experiments reported above do not permit conclusions concerning all the details bringing about inhibition by these drugs, but it is assumed that iodine absorption is only one of the many steps in the manufacture of thyroid hormone which is in some way hindered. A more complete understanding of the processes controlling normal thyroid function would aid greatly in the interpretation of the whole mechanism of the goitrogenic effect of these compounds.

Summary

1. The iodine-concentration capacity of the thyroid gland, as measured by the uptake of radioactive iodine, was depressed in frogs injected with thiourea, sulfanilamide and potassium thiocyanate. The subcutaneous injection of 0.5 cc. of a 10 per cent solution of thiourea and of sulfanilamide, and 1 cc. of a 0.2 per cent solution of potassium thiocyanate into normal frogs resulted in an enlarged, hyperemic and hyperplastic thyroid gland. The thyroid hyperplasia which occurred under the influence of these drugs was a secondary compensatory response to the increased thyrotropic hormone output by the hypophysis. Inhibition was most pronounced with thiourea, sulfanilamide and potassium thiocyanate following in that order.

2. Injected potassium thiocyanate was rapidly removed from the blood stream, and its elimination was practically complete in 24 hours. However, the iodine-concentrating capacity of the thyroid gland, as measured by the uptake of radioactive iodine, was depressed by potassium thiocyanate, provided a high concentration of the drug was still present in the circulation. After 24 hours, the enlarged thyroids of the potassium thiocyanate-treated frogs had a greater capacity than normal for absorbing injected radioactive iodine.

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STUDIES OF THE DAILY RHYTHMICITY OF THE FIDDLER CRAB, UCA. MODIFICATIONS BY PHOTOPERIOD[1]

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Since the first report of a persistent diurnal chromatophore rhythm in Uca by Abramowitz (1937), considerable work has been done in this laboratory as well as others in an attempt to elucidate the mechanisms controlling this periodic response. These studies, together with the reports of workers concerned with other manifestations of diurnal periodicity of function, have produced a literature which is extensively reviewed in Welsh (1938) and Webb (1950).

Light is the most obviously diurnally periodic feature of most environments and for this reason has been a popular experimental variable in studies of the control of diurnal rhythms. However, despite this fact, no work has been reported studying the effect of varying the extent of the daily photoperiod on the quantitative relations of a diurnally periodic response. The diurnal chromatophore rhythm of Uca can be readily observed and use of the Hogben-Slome scale for measuring chromatophore dispersion allows an accurate quantitative description of the characteristics of this rhythm. These characteristics make Uca a suitable experimental animal for such purposes. Thus the experiments to be reported were designed to contribute to an understanding of the mechanisms controlling diurnal periodicity by studying the character of the responses of this chromatophore rhythm to variations in the length of the daily light period to which the animals are exposed.

Material and Methods

All animals used in these experiments were male specimens of *Uca pugnax* collected in the vicinity of Woods Hole, Massachusetts. The animals were placed in white enameled pans in groups of twenty and subjected either to illumination at an intensity of 100 foot candles or to darkness, depending on the protocol which the group concerned was to follow. This was done not later than two hours after collection. The time of collection was between 10 A.M. and 4 P.M., this time being chosen to avoid the dark period of as many of the experimental groups as possible in order to provide constant experimental treatment.

Two sets of experiments are to be reported which differ to some extent in protocol. All succeeding references to light refer to light at an intensity of 100 foot candles furnished by the laboratory fluorescent lights. Groups were subjected to indicated periods of darkness by placing them in an adjacent darkroom. In both sets of experiments the condition of the black chromatophores in each experimental group was determined at 1, 4, 7, and 10 A.M., and 1, 4, 7, and 10 P.M. by

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recording the extent of dispersion for each animal in terms of the Hogben-Slome scale.

The first set of experiments was begun on July 26, 1950 and terminated on August 9, 1950. The following groups were maintained and the black chromatophore activity of each ascertained in the manner described above.

1) A control group maintained in constant darkness from 4 P.M. July 26 until the experiments were concluded.
2) A group exposed to light from 7 A.M. to 1 A.M. and darkness from 1 A.M. to 7 A.M. daily (18-hour photoperiod).
3) Exposed to light from 1 A.M. to 7 P.M. (18-hour photoperiod).
4) Exposed to light from 4 A.M. to 10 P.M. (18-hour photoperiod).
5) Exposed to light from 7 A.M. to 1 P.M. (6-hour photoperiod).
6) Exposed to light from 1 P.M. to 7 P.M. (6-hour photoperiod).
7) Exposed to light from 10 A.M. to 4 P.M. (6-hour photoperiod).

In each case not specified above, the animals concerned spent the remainder of each 24 hour period in darkness and were accorded the same treatment daily for the duration of this portion of the experiments. On July 31 groups 2 through 7 above were placed in darkness after exposure to light as outlined above and so maintained until the experiments were terminated on August 9. There are two exceptions to this statement; one-half the animals remaining in groups 3 and 4 were placed in darkness at 1 P.M. and 4 P.M., respectively, on July 31. It was found that this treatment altered neither the amplitude of the rhythm nor the magnitude of the dispersed phase and produced only a transient effect on the time of concentration. After this had been ascertained, these animals were discarded and are not included in the calculation of the magnitude of the dispersed phase for the groups which will be discussed subsequently. Thus, at the end of this set of experiments, groups 3 and 4 were smaller than the others, containing respectively seven and five animals. In no other case did less than 10 of the original 20 animals in each group survive the experimental period.

The second set of experiments was begun on August 14, 1950 and terminated on August 24, 1950. The following groups were maintained and black chromatophore activity ascertained in each case as before.

1) A control group maintained in constant darkness as before for the duration of the experiments.
2) A second control group was maintained in conditions as closely approximate to normal with respect to light periodicity as feasible. The animals were exposed to daylight in a wooden collecting bucket. No attempt was made to control or measure intensity but the animals were shielded from direct sunlight to avoid lethal temperatures. Sea water in the bucket was renewed daily and the animals were shielded from rain when necessary.
3) Exposed to light from 4 A.M. to 10 P.M. daily (18-hour photoperiod).
4) Exposed to light from 10 A.M. to 4 P.M. daily (6-hour photoperiod).

On August 18, groups 3 and 4 were placed in darkness after their respective exposures to light (in the case of group 2 at 1 A.M. on August 20) and so maintained until the experiments were terminated. In no case did less than 9 of the original 20 animals in each group survive the experimental period.

The diurnal rhythm of the black chromatophores persisted in all cases. For the purposes of analysis of the data this rhythm has been treated as a daily excursion into the dispersed phase and direct measurements of the course of each 24 hour cycle transformed into a parameter which is proportional to the area swept out by the dispersed phase. The formula used for this calculation is

$$A = \frac{\sum_{i=1}^{n} (x_i - 1)}{8}$$

"i" refers to the time of the reading concerned. "i = 1" was arbitrarily taken as the initial point of minimum dispersion in the cycle under consideration.

"x_i" is the average extent of dispersion of the black chromatophores of the group concerned as measured in terms of the Hogben-Slome scale at the time "i".

"n" is the number of the reading which indicates the second point of minimum dispersion and the conclusion of the cycle considered. The magnitude of "n" varied between 8 and 10. Reading "n" for one cycle is then reading "1" for the cycle immediately succeeding.

This formula essentially gives an expression for the average dispersion of the black chromatophores in the course of one cycle, on the assumption that each cycle occurs in 24 hours. This assumption is thoroughly justified on the basis of the observational data.

RESULTS

The values for the average dispersion of the black chromatophores for each group calculated, using the formula explained above, are arranged in tabular form

TABLE I

Daily magnitude of the dispersed phase in each group of the first series of experiments. All groups in constant darkness after the fifth day

Group......... Day	1	2	3	4	5	6	7
1	2.10	2.33	2.45	2.39	1.63	1.81	1.94
2	2.36	2.45	2.66	2.66	1.76	2.00	2.11
3	2.71	2.46	2.65	2.64	2.04	1.81	2.09
4	2.50	2.64	2.61	2.64	2.16	2.07	2.21
5	2.32	2.69	2.76	2.81	2.11	1.89	2.14
6	2.56	2.57	2.23	2.56	2.26	1.99	2.11
7	2.53	2.23	2.29	2.28	2.16	1.93	2.13
8	2.51	2.25	2.29	2.35	2.28	2.36	2.09
9	2.89	2.49	2.32	2.50	2.16	1.99	2.14
10	2.61	2.45	2.41	2.06	2.20	2.23	2.10
11	2.46	2.29	2.20	2.05	1.97	1.76	1.98
12	2.53	2.39	2.21	2.18	2.18	2.02	1.96
13	2.58	2.50	2.39	2.13	2.02	2.06	1.85
Av. (6–13)	2.58	2.40	2.30	2.26	2.16	2.04	2.04
SD	±.08	.11	.06	.16	.08	.13	.09
Aggregate average		2.32 ± .14			2.08 ± .11		

in Tables I and II. Inspection of the values for the first five days of the first series of experiments as presented in Table I is sufficient to validate the statement that for this period the responses of groups 2, 3, and 4 are similar and differ significantly from the responses of groups 5, 6, and 7. Further, the responses of groups 5, 6, and 7 are similar. The antepenultimate row of Table I lists the average value of the magnitude of the dispersed phase in cycles 6 through 13 of the groups concerned and the penultimate row gives the standard deviation for this value. These cycles were chosen as representative of what may be called the resting level of the rhythm in constant darkness. It is seen that these average values fall naturally into three sets comprised of group 1, groups 2, 3, 4, and groups 5, 6, 7. The last row of Table I lists the averages of cycles 6 through 13 for groups 2, 3, 4, and groups 5, 6, 7 considered as aggregates, together with the standard deviation of these values. This constitutes evidence for the conclusion that the magnitude of the dispersed phase in the rhythm, both during the periodic light exposure and in constant darkness afterwards, is influenced by the length of daily exposure to light but is uninfluenced within the limits of the experimental procedure by the time of day at which the animals are exposed.

Table II

Daily magnitude of the dispersed phase in the second series of experiments. All groups in constant darkness after the fifth day except 2 which was placed in darkness on the sixth day

Group............... Day	1	2	3	4
1	1.35	2.30	2.23	1.59
2	1.38	2.11	2.44	1.79
3	1.59	2.01	2.54	1.69
4	1.80	2.13	2.48	1.80
5	1.95	2.06	2.65	1.84
6	2.18	2.20	2.19	1.84
7	2.11	1.54	2.33	1.94
8	2.13	1.41	2.32	1.79
9	2.18	1.59	2.16	1.73
10	1.99	1.80	2.09	1.67
Av. (6–10)	2.12		2.22	1.79
Av. (1–6)		2.13		
SD	±.05	.08	.09	.08

Table III lists the deviations of the minimum dispersion point at the start of each cycle for each group from an arbitrary zero point of 1 A.M. Two conclusions are supported by these tabulated data. On July 26, 1950, time of sunrise was 0530 Eastern Daylight Saving time and time of sunset was 2008. This places the middle of the naturally-occurring dark period at −0.18 in terms of the units used in Table III. Similar considerations place the middle of the naturally-occurring dark period on August 14, 1950 at −0.23. Given these facts, reference to the average values in the last row of Table III for series I, group 1 and series II, groups 1 and 2 allows the conclusion that the point of minimum dispersion does not normally coincide with the middle of the natural dark period nor does it bear any simple fixed relation to it. Evidence is provided for a second conclusion by inspection of the average deviations for series I, groups 2 and 3. Both groups were exposed to light for 18 hours per day but the middle of the experimental dark period was at 4 A.M. and 10 P.M. respectively. The respective increments with respect to the middle of the normally-occurring dark period on July 26 are 3.18 and −2.82. The measured increments are 2.96 and −1.86. That the induced shifts are permanent is attested by the fact that the averages of points 6 through 14 (*i.e.,* after the animals were in constant darkness) are 2.67 and −2.33 in the two cases, manifesting no tendency to drift back to the control value represented by group 1 in this series. The value for series II, group 3 is further evidence for the existence of a persistent shift. It should be pointed out that since measurements were made at three-hour intervals, the error for each of the tabulated values is high, of the order of an hour and a half. However, the probable error of the average of a number of such values is considerably smaller and does not vitiate the conclusions drawn.

TABLE III

Daily deviations of the point of minimum dispersion from 1 A.M. as an arbitrary zero point. Time in hours

	Series I							Series II			
Group... Day	1	2	3	4	5	6	7	1	2	3	4
1	0	3.0	0	0	0	0	0	0	−3.0	1.5	−1.5
2	0	3.0	−1.5	0	1.5	1.5	1.5	1.5	0	0	−1.5
3	1.5	3.0	−1.5	1.5	3.0	3.0	3.0	−1.5	0	0	−1.5
4	0	3.0	−1.5	0	−1.5	0	0	0	−3.0	0	−1.5
5	3.0	3.0	−3.0	0	−1.5	−3.0	0	−1.5	−1.5	0	−1.5
6	0	3.0	−1.5	0	−1.5	0	−1.5	−1.5	−1.5	0	−3.0
7	3.0	3.0	−3.0	−3.0	−3.0	3.0	0	−1.5	−1.5	0	0
8	1.5	1.5	−3.0	−3.0	0	0	0	−3.0	−1.5	0	−1.5
9	0	1.5	−1.5	−3.0	0	0	0	−1.5	0	0	−1.5
10	1.5	3.0	−3.0	−1.5	0	0	0	−3.0	−1.5	0	−3.0
11	1.5	3.0	−3.0	−3.0	1.5	3.0	0				
12	0	3.0	−3.0	−1.5	−1.5	−3.0	−1.5				
13	0	3.0	−3.0	−1.5	0	0	0				
14	0	3.0	0	0	−1.5	0	0				
Av.	.86	2.78	−2.04	−1.07	−.32	.32	.11	−1.20	−1.35	.15	−1.65

Figure 1 displays graphically the variation in the magnitude of the periodic dispersion of the black pigment in the first series of experiments. It has been previously argued that the time of day at which the animals were exposed to light was immaterial in a consideration of induced shifts in this magnitude, so that the data from groups 2, 3, and 4 have been averaged to give the points representing the response of animals exposed to 18 hours of light daily and the data from groups 5, 6, and 7 contribute in the same way to the curve for animals exposed to 6 hours of light daily. Figure 2 presents the same information for groups accorded similar treatment in the second series of experiments and in addition records the variations in dispersion for the additional control group maintained under normal light conditions.

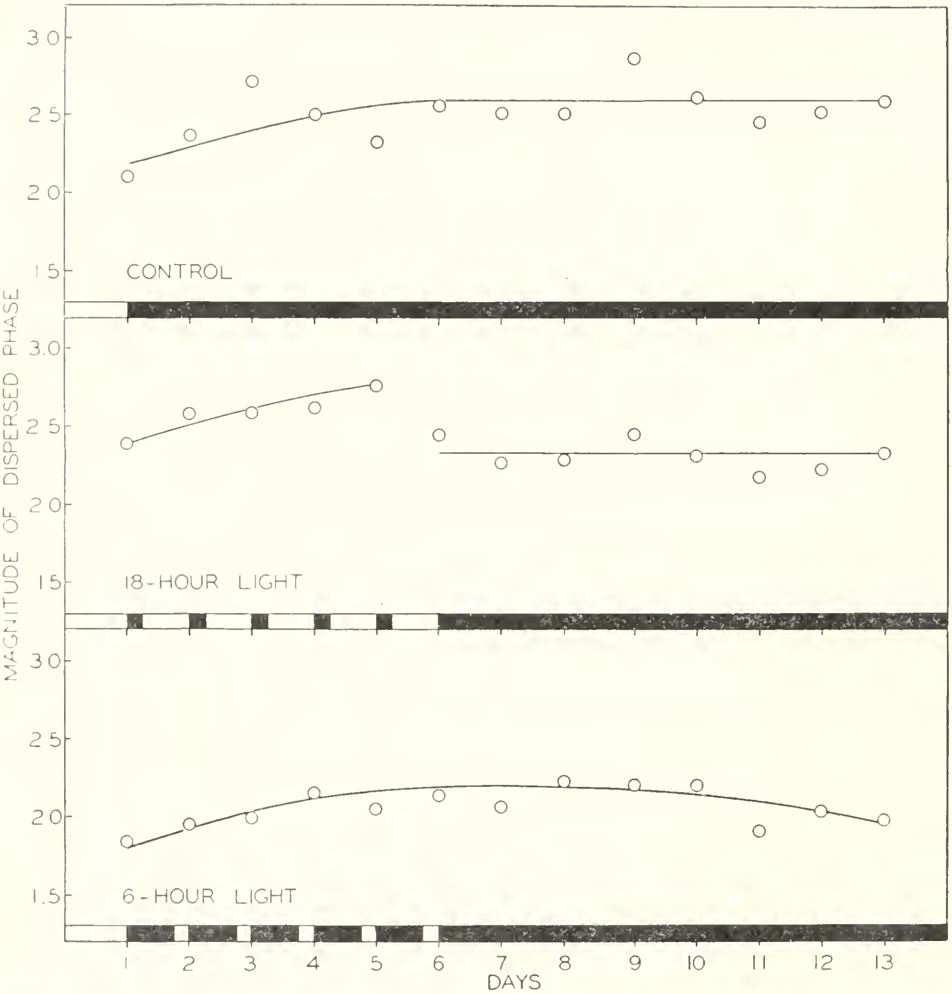

FIGURE 1. The responses of the magnitude of the dispersed phase of the chromatophore rhythm in the three groups of the first series of experiments. The white and black blocks under each curve are a diagrammatic representation of the relative lengths of each 24 hour period spent in light and darkness respectively.

There are several features of these graphs which are of interest. It will perhaps be simplest to discuss the responses of the groups receiving similar treatment in the two sets of experiments.

One of the most striking features of the control values in both figures is their smooth increase over a period of several days from an initial relatively low magnitude to attain finally a resting level. This is particularly pronounced in series II (Fig. 2) where the effect manifested by the controls in darkness is repeated almost quantitatively by the controls maintained in normal daylight when placed in constant darkness after the sixth day. This portion of the response is less evident in series I

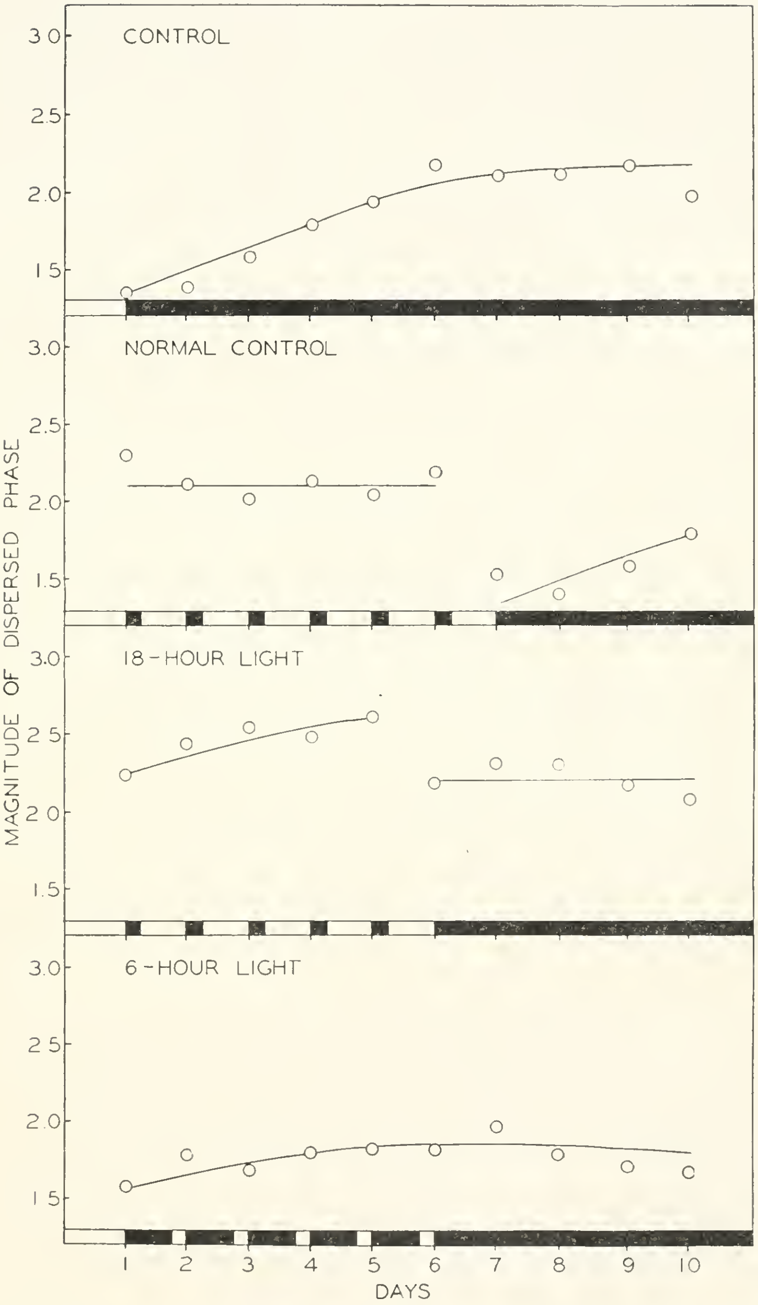

FIGURE 2. The responses of the magnitude of the dispersed phase of the chromatophore rhythm in the four groups of the second series of experiments. The white and black blocks beneath each curve have the same significance as in Figure 1.

although it can be discerned. It is of even greater interest in that no similar depression seems to intervene between the response while receiving a daily light ration and the subsequent resting level in darkness in either of the 18-hour or the 6-hour groups. Another point of interest is the correspondence between the resting level of the control group kept in darkness and the level maintained by the controls kept in normal daylight conditions. The average values and standard deviations for the two levels are 2.12 ± 0.05 and 2.13 ± 0.08 respectively. In the light of the character of these experiments, it would be of the greatest interest to be able to state the day-length to which the animals collected were exposed under natural conditions. The times of sunrise and sunset for July 26 and August 14 have been reported above. If these values be taken as the initial and final points of the light period, the day-length in the two cases is 14 hours 38 minutes and 13 hours 58 minutes, respectively. However, if the time for the beginning of morning twilight and end of evening twilight, as listed in American Ephemerix and Nautical Almanac for 1950, be taken as initial and final points, the day-lengths on the two days become 18 hours 39 minutes and 17 hours 28 minutes.

Animals exposed to 18 hours of light per day (series I, groups 2, 3, 4, and series II, group 3) display an increasing dispersion of the black chromatophores. This increase is apparently not immediately induced but represents a response extending over a period of several days. The initial value in both series is near the resting level of the control groups so that we may conclude that this cumulative increase operates on an initially normal value (see preceding paragraph) producing a characteristic smooth rise. As previously mentioned, in neither of the sets of experiments does a depression intervene between this phase of the response and the assumption of a resting level in darkness. It is further of interest that the levels assumed in the two cases are quite similar despite large discrepancies in the control and 6-hour experimental levels.

Animals exposed to 6 hours of light per day (series I, groups 5, 6, 7 and series II, group 4) manifest an initial depression (below resting level) and then rise steadily in the course of three or four days to assume a level which remains unchanged with the advent of continuous darkness. This level is significantly below the control level in both cases but appears to differ by about 0.2 units in the two sets of experiments.

Discussion

If we first consider the problem of the control of the magnitude of the periodic dispersion of the black chromatophores, several lines of reasoning indicate that two more or less independent controlling centers must be postulated. Let us first examine the response of animals exposed to 18 hours of light daily. The initial phase of the response is a modification of the rhythm which has as its starting point the normal magnitude. The cumulative character of this phase of the response indicates that it is not to be explained as a direct response (where "direct" is used to refer to a response not mediated by a rhythmical center whose activity is influenced by one or more types of environmental stimuli). The fact that the level subsequently assumed by the animals receiving this treatment in series I is significantly below the control level, although the values attained in the initial phase of the response are significantly above this level, indicates that the center whose activity was cumulatively modified to produce the initial increment is not responsible for the level at which the rhythm stabilizes in continuous darkness.

An examination of the behavior of the control groups furnishes further support for the hypothesis of a dual control of chromatophore rhythmicity. The period of depression or inhibition evident in the response of all groups on being placed in constant darkness from normal conditions is smoothly released, indicating again a cumulative modification of the activity of a controlling center. That this is not the controlling center responsible for the resting level assumed in darkness is indicated by the significant difference between the resting level of the control groups in the two sets of experiments.

Let us consider the characteristics of the two centers thus supported and attempt to describe insofar as possible the stimuli by which the activity of each is modified and the relations between the two centers. We have then two centers, A and B, which are concerned with the control of the diurnal periodicity of the black chromatophores of Uca. If the following characteristics are postulated, an explanation of the reported results can be given.

[illegible] in length

1) Center A manifests a persistent 24 hour rhythmicity in the secretion of a black-dispersing principle and the extent of this secretory activity is modified only by changes in the length of the daily photoperiod to which the animal is exposed. In addition to a direct effect on the stage of black chromatophore dispersion, this secretion, or another concomitant activity of center A, exerts a controlling influence on the rhythmical activity of center B. Total darkness or a daily photoperiod below some threshold length (no experimental evidence is available to permit a choice between these alternatives) is not effective as a stimulus in changing the activity of center A. seasonal
2) Center B also manifests a 24 hour rhythmicity but a more labile one than center A, and less persistent. Center B gradually becomes completely synchronized with center A when environmental stimuli which normally influence center B are removed. Center B has as mediating secretions both a black-dispersing and a black-concentrating substance. It responds to darkness by secretion of the black-concentrating substance and to light by secretion of the black-dispersing substance at its disposal. More precisely, there is an intensity *i* such that at or below this intensity a black-concentrating principle is secreted, this response being graded, and there is another intensity *j*, which may or may not be identical with *i*, such that at or above this intensity a black-dispersing principle is secreted, this response also being graded. — Daily (cloudy sunny)

With two centers having these characteristics, it is possible to give a coherent explanation of the results of the two series of experiments. Control groups, it will be remembered, responded to being placed in total darkness by an initial depression in magnitude with a gradual return to an original resting level. Since there were no stimuli which could effect a change in the activity of center A, and since centers A and B would normally be acting synergistically, it is reasonable that the resting level in darkness should equal the level maintained in normal daylight. Further, since center B would respond to darkness with an increased secretion of a concentrating principle, a depression of the rhythm magnitude is to be expected until A regains complete control of the periodic activity of B.

When animals are placed in 18 hours of light at an intensity of 100 foot candles the situation is somewhat complicated. Under normal conditions, the animal would be subjected to periods of low intensity at the beginning and at the end of the photoperiod, from which it follows that center B would be activated to produce a dispersing substance during only a portion of the normal day-length exposure. However, under experimental conditions the animals were exposed to an intensity of 100 foot candles throughout the photoperiod. Thus, B was activated to produce a dispersing substance at a time when it had not been so activated under natural conditions. Such activation modified not only the immediate response of B but the character of its rhythmical activity so that as the stimulus was repeated on successive days, the reaction of center B to light became more and more effective in producing chromatophore dispersion. Thus, we observed a cumulative increase in magnitude as the initial response of these groups. Although center B continued its increased activity in the production of dispersing substance after the animals were placed in continuous darkness, this in itself also operated as a stimulus to the production of a black-concentrating substance. Both of these effects would be transient as center A gradually established control of the activity of B, and since they are antagonistic, only one of them could be expected to be observed and it should be less pronounced than it would be if operating alone. In fact, neither trend is observable. On the basis of this hypothesis, since the natural photoperiod on July 26 was greater than 18 hours (18 hours 39 minutes), center A should stabilize the rhythm at a level somewhat below the control level. The natural day-length on August 14 being 17 hours 28 minutes, the resting level in 18-hour animals should exceed that for the controls. Both of these expectations are confirmed. It should be pointed out in this connection that there is no reason to expect the activity of center A to be linearly related to the length of the photoperiod, so that it suffices to show one effect is greater than another, provided the stimulating day-length for the first is longer without regard to quantitative correspondence of increments. Finally, it is reasonable that the resting level attained by the 18-hour animals in darkness should be the same despite discrepancies in control level if center A is responsible for the control of this response.

In the response of animals exposed to 6 hours of light daily, center B is responsible for the initial drop in magnitude by its secretion of concentrating substance in response to the increased darkness and this effect is later supported by the induced shift in activity of center A. However, since center A responds slowly to changes in the length of the light period, the total effect will be a slow rise mimicking that shown by control groups when placed in darkness. For the same reasons which were adduced in the discussion of the reaction of the 18-hour animals, either an increase or a decrease in magnitude could be produced when the animals were placed in continuous darkness and neither was observed (the decrease observed in both sets in the course of several days is probably too small to be significant). On the basis of this hypothesis it is reasonable that the levels in the two cases were not the same since, by hypothesis, center A responds slowly to a change in light period. This argument does not apply to the case of the 18-hour animals since the experimental value in that case was quite close in both sets of experiments to the natural photoperiod.

Centers A and B discussed in the preceding hypothesis are in no way incompatible with centers 1 and 2, respectively, of Brown and Webb (1949), which were postulated to account for other responses of the rhythmic mechanism of Uca; in fact, they even exhibit the same intrinsic characteristics. They are, therefore, assumed to be the same and this work is considered to provide striking support for the earlier hypothesis and to describe further properties of these two postulated centers.

Figure 3 is a diagrammatic representation of the mechanism which has been hypothesized to account for the responses of the daily rhythmicity observed during these experiments. In conformity with the earlier work, centers A and B are here indicated as 1 and 2.

With regard to the elements involved in this hypothesis, there is no reason to be concerned with the identity or non-identity of the dispersing principles whose liberation is controlled by A and B. The hypothesis requires only that there be two controlling centers, each of which has at its disposal a dispersing substance. Although it is not necessary that these two centers be physically discrete, it is possible that they are. Thus, it is of interest that both the sinus gland and the central nervous system in Uca elaborate black-dispersing substances (Sandeen, 1950). There is no direct evidence for the existence of a black-concentrating principle but other work done in this laboratory (Brown and Hines, in press) provides indirect support for its postulation.

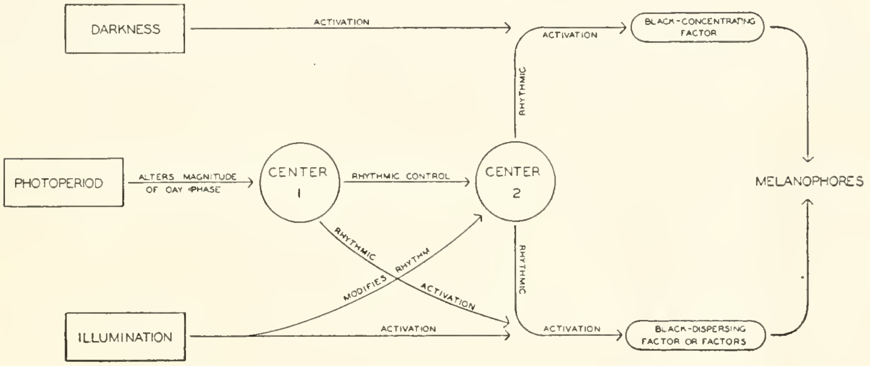
DARKNESS
ACTIVATION
ACTIVATION
BLACK-CONCENTRATING FACTOR
RHYTHMIC
PHOTOPERIOD
ALTERS MAGNITUDE OF DAY PHASE
CENTER 1
RHYTHMIC CONTROL
CENTER 2
MELANOPHORES
RHYTHMIC ACTIVATION
MODIFIES RHYTHM
RHYTHMIC
ILLUMINATION
ACTIVATION
ACTIVATION
BLACK-DISPERSING FACTOR OR FACTORS

FIGURE 3. A diagrammatic representation of the postulated control mechanism of the black chromatophore rhythm of Uca.

The complexity of this controlling mechanism leads inevitably to speculation concerning the reasons for such elaborate systems. The idea has often recurred in the invertebrate endocrine literature that chromatophorotropins are simultaneously concerned with more basic functions. If, purely speculatively, this reasoning is followed, one may ask what functions would be thus controlled by the photoperiod. Panouse (1943, 1944, 1946), Brown and Jones (1949), and Stephens (1951) have demonstrated clearly the endocrine nature of the control of ovarian maturation, and the last investigator has shown a role of photoperiod in the decapod sex cycle. We have here a function which is presumably dependent on responses to change in day-length but which also requires that the responding system be uninfluenced by large short term variations in photoperiod. On the other hand, Edwards (1950) and others have pointed to the significance of endocrines in the diurnal activity rhythm of the decapods. This, in contrast, requires an elastic response system capable of following short term variations. It is obvious that two such functions, if combined, produce a total chromatophorotropin system similar to the one described. Center A in our hypothesis represents an inelastic response mechanism whose characteristics are such as to make it suitable for the control of the sex cycle. Center B possesses the necessary lability for control of a function such as locomotor activity rhythm which must be warped to the exigencies of short-term environmental variations. Although there is some scattered evidence that some chromatophorotropins have other systemic effects in the decapods, such evidence can afford only general support for the possibility of this kind of interrelation which must remain speculation until further experimental evidence is accumulated. On the other hand, it is quite possible that these same two centers will be shown to exert their influences directly upon other nervous and endocrine mechanisms than those involved in color changes.

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Table III provides evidence that permanent shifts in the relation of the phases of the diurnal rhythm to solar time, of the order of two to three hours in either direction from an arbitrary zero point, can be induced by suitable variation in the time of illumination of the animals. The data seem insufficient to warrant a more extended analysis or to validate less general conclusions, but merely indicate the desirability of a more refined approach to the problem of the control of the temporal relations of the diurnal chromatophore rhythm.

SUMMARY

1. It has been shown that if the diurnal rhythm of the black chromatophores of Uca is treated as a periodic excursion into the dispersed phase, the magnitude of this excursion is significantly influenced by the length of the daily photoperiod to which the animals are subjected.

2. That persistent alterations are induced by different photoperiods is clearly seen in the levels at which the rhythm stabilizes after the animals are placed in constant darkness subsequent to experimental treatments with light.

3. The time of an 18-hour period of illumination between 1 A.M. and 1 A.M. appears not to influence the induced magnitude of the rhythm. The time of a 6-hour period of illumination between 7 A.M. and 7 P.M. is similarly immaterial. However, it has been shown that variations in the time of day within these limits of the period of illumination can produce corresponding and persistent shifts in the relation of the phases of the rhythm to solar time. These alterations may be in either direction from an arbitrary zero point.

4. In addition to the induction of persistent modifications of the rhythm by photoperiod, the character of the rhythm has been shown to be influenced also by illumination and by darkness. These latter effects are transient when the animals are no longer exposed to alternating periods of light and darkness.

5. An analysis of the data clearly indicates the operation of two centers of rhythmicity, such that the activity of the first is influenced by the length of the photoperiod to which the animal is exposed while the activity of the second is influenced by the duration and intensity of light to which the animal is exposed daily, by the duration of the daily dark period, and by the activity of the first center.

6. An adaptive significance for this duality of the control of the rhythm is suggested.

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THE UPTAKE OF VANADIUM BY TUNICATES[1]

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The ability of certain ascidians to concentrate vanadium was first reported in 1911 by Henze (1911), who found this element in the blood of four species. Since then more than a score of papers have extended these investigations.[2] The scope of the above work has included the analyses of various species of ascidians to determine the presence or absence of vanadium, and experiments to indicate any possible physiological function of the vanadium.

The first phase of these studies culminated in the work of Webb (1939) who was able to correlate the presence of vanadium in ascidians with certain evolutionary traits of the class. Webb concluded that vanadium is concentrated by the primitive species and that this enrichment ability has been lost in the later, more specialized, branches of the class.

Webb also systematically studied the physiological role of vanadium. He demonstrated that the green chromogen containing the vanadium in some species is located in a singular type of blood cell which he called the "vanadocyte." Later, however, in histological investigations on certain Japanese ascidians, Ohuye (1936) described what may be the same type of green cells in these animals which, according to the analyses of Kobayashi (1948), contained no vanadium. Webb found no physiological function for the vanadium, although previously it had been assumed to be associated with a blood respiratory pigment. He also postulated that the vanadium was incorporated in a rectilinear chain of pyrrole rings rather than in a protein or porphyrin complex.

The reported distribution of the vanadium within ascidians is somewhat contradictory. Webb suggested that all of the vanadium is located in the blood, whereas the experiments of Kobayashi and of Bertrand localized the majority of the vanadium in other parts of the animal. In *Ascidia mentula* Bertrand found ⅔ of the vanadium in the tunic, yet Kobayashi was able to establish only traces of vanadium existing in the outer covering of Japanese ascidians.

The dynamics of the concentration of vanadium from sea water by these marine animals has received scant attention. Bertrand describes the situation as a complete mystery and states that the vanadium could come neither from sea water, which is very poor in vanadium, nor from vanadium-impoverished marine muds in the vicinity of the ascidians. He invites research on plankton as a potential source of vanadium. Rankama and Sahama (1950) suggest, however, that the vanadium is collected by ascidians directly from sea water.

Thus, from a biogeochemical standpoint it seemed desirable to investigate the more intimate details of the concentration of vanadium by marine organisms. The present investigation was undertaken in order to attempt to follow the transfer of vanadium from the sea into the various parts of the body of ascidians. A knowledge of the mode of uptake and its histological distribution might shed light upon a possible role played by the vanadium.

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[1] Contributions of the Scripps Institution of Oceanography, New Series No. 528.

[2] A comprehensive survey of the biogeochemistry of vanadium has been prepared by D. Bertrand (1950).

To lay the groundwork for these investigations, chemical analyses of dissected portions of some local ascidians were made. This work was intended to establish more definitely the sites of vanadium concentration. A limited number of analyses were made on the total vanadium content of some local species for inclusion in Webb's phylogenetic table.

Cyclotron-produced radioactive vanadium, free from any inert vanadium carrier, was utilized to follow the assimilation of the element. Radioautographs were made to confirm the results of the chemical analyses and also to point out any smaller loci of vanadium concentration not detected by the less sensitive chemical method.

The authors wish to express their appreciation for the aid given by Dr. Denis L. Fox, Professor of Biochemistry at the Scripps Institution of Oceanography, for his help in the completion of this problem.

CHEMICAL ANALYTICAL TECHNIQUES

Specimens of *Ciona intestinalis* were collected from floats in Mission Bay, San Diego, California. The *Ascidia ceratodes* specimens were obtained from dredgings in Tamales Bay, Marin County, north of San Francisco, California.

TABLE I

Vanadium content of Ciona intestinalis

Specimen A			Specimen B		
Sample	Dry Wt.	Vanadium	Sample	Dry Wt.	Vanadium
	grams	γ		*grams*	γ
Tunic	0.1629	0.0	Tunic	0.3317	0.0
Mantle	—	—	Mantle	0.0709	0.0
Ovary	0.0330	2.5	Ovary	0.0241	0.0
Heart	0.0084	0.0	Heart	0.0113	0.0
Incurrent Syphon	—	—	Incurrent Syphon	0.0368	4.0
Excurrent Syphon	—	—	Excurrent Syphon	0.0228	0.0
Esophagus	—	—	Esophagus	0.0014	0.0
Branchial Basket	—	—	Branchial Basket	0.0241	0.0
Gut			Gut		
Anterior Half	0.0517	19.7	Anterior Half	0.1116	21.9
Posterior Half	0.1375	8.9	Posterior Half	0.1252	37.8
Stomach	0.0543	18.8	Stomach	0.0586	9.2
Rest of Specimen and Rinse	0.2299	27.0	Rest of Specimen and Rinse	—	—
Rinse		—	Rinse	0.2955	19.6
Total	0.6777	76.9	Total	1.1140	92.5
Date Ciona Obtained	24 July 1950		Date Ciona Obtained	20 April 1950	
Wet Weight	19.0 grams		Wet Weight	19.0 grams	
Length	8.0 cm		Length	6.5 cm	

TABLE II

Total vanadium contents of some ascidians

Ascidian	Parts Vanadium Per Million
Styella montereyensis	0
Styella bonharti	0
Euherdmania claviformis	475
Ciona intestinalis	100

The specimens were cleaned of adhering dirt and sessile organisms by the light application of a tooth brush. After dissection the individual samples were placed in weighed 50 ml. Pyrex centrifuge tubes. The tube and contents were dried overnight at 110° C., cooled in a desiccator, and weighed to determine the "dry weight" of the sample.

The organic matter was removed by a wet digestion as follows: to the sample-containing tube, 3.0 ml. of a solution of one part concentrated sulfuric acid to three parts of 60 per cent perchloric acid were added. The tube was heated gently with a micro-burner until the sample was nearly completely digested. Stronger heat was then applied to facilitate the complete removal of the perchloric acid. The sulfuric acid residue was diluted to 40 ml. with distilled water, and any remaining solids (usually diatom frustules) were removed by centrifugation.

The vanadium in the clear solution was determined by a spectrophotometric analysis of the vanadium 8-hydroxyquinolate in iso-amyl alcohol according to the method of Bach and Trelles (1941). The samples were read at 480 mμ in 50 mm. cells on a Beckman Model DU Spectrophotometer.

The results of two typical analyses of *Ciona intestinalis* are summarized in Table I. The vanadium concentrations for other ascidians collected during the course of these investigations are listed in Table II.

Relatively high concentrations of vanadium occur in the gut. The gut tube was arbitrarily dissected into four serial lengths. It was found that the highest vanadium concentrations per unit weight were associated with that part of the gut nearest the stomach, and with the segment adjoining the anus (Table III). Vanadium was normally detected in the stomach by these chemical analyses.

Vanadium was also found in fecal pellets in concentrations ranging from 0 to 800 parts per million by weight. Fecal pellets discharged by the animal during its relaxation before dissection, or dislodged during the dissection, may account for the high vanadium content of the rinse water as noted in Table I.

TABLE III

Concentration of vanadium in gut sections of Specimen B

Section	Length	Dry Weight	Vanadium
1 Next to Stomach	10 mm.	0.0601 gr.	19.3γ
2 Next segment	10 mm.	0.0515 gr.	2.6γ
3 Next segment	10 mm.	0.0601 gr.	11.8γ
4 Next to Anus	10 mm.	0.0651 gr.	26.0γ

FEEDING EXPERIMENTS UTILIZING RADIOACTIVE VANADIUM

V^{48}, a positron and gamma ray-emitting nuclide with a half-life of sixteen days, was prepared by the deuteron bombardment of a titanium target according to the reactions $Ti^{47}(d,n)V^{48}$ and $Ti^{48}(d,2n)V^{48}$. The bombardments were made at the 60-inch Crocker Cyclotron in Berkeley, California, with 20 Mev deuterons for one hour with a total irradiation of 7 micro-ampere hours. The titanium target was composed of one gram of the C.P. metal powder supported on a copper plate covered with a 0.25 mil tantalum foil.

The sub-micro amounts of V^{48} were extracted from the titanium powder to give a carrier-free saline solution of radioactive vanadate, following the method of Haymond, Maxwell, Garrison and Hamilton (1950). The nuclide V^{48} was identified by its half life and by aluminum absorption curves to establish the 0.7 Mev positron reported by Peacock and Deutsch (1946).

Individual ascidians were then placed in aerated Pyrex containers which held two liters of sea water. These aquaria were kept in a cold room maintained at a temperature of 12° ± 2° C.

The sea water was normally filtered before use. In preliminary experiments it was found that the radioactive vanadium was adsorbed on particulate matter in non-filtered sea water and was thus removed from solution. The water was filtered through either Whatman No. 42 paper or a diatomaceous earth column. The radioactive vanadium was added after filtration.

The activity of the solution was assayed daily by evaporating under an infrared lamp a one-ml. aliquot of the sea water in a steel cup. The activity of the sample was counted under an end-window tube with a window thickness of 1.4 mg./cm^2.

A control aquarium was set up without an ascidian to determine whether there was adsorption of the radioactive vanadium on the walls of the container. No measureable adsorption was found to take place.

The initial counting rate of the radioactive solution was about 250 counts per minute per ml. of solution. The counter geometry was estimated at 5 per cent for 0.7 Mev positrons from measurements with a calibrated U_3O_8 source and aluminum absorption experiments on the active vanadium. Utilizing the radioactive decay equation,

$$dN/dt = -\lambda N \tag{1}$$

where N is the number of atoms of the radionuclide present, λ is the decay constant equal to 0.693 divided by the half life, and dN/dt is the instantaneous disintegration rate, 10^{11} atoms of active vanadium are found to exist in one liter of solution initially. This may be compared with the upper limit of vanadium in sea water of 10^{16} atoms per liter as determined spectroscopically by D. Bertrand (1942). It should be noted, however, that the experimentally determined impurity of 0.01 per cent of vanadium in the one gram of titanium metal target introduced about 10^{16} atoms of vanadium when it was diluted to 100 liters in the experiments that were undertaken.

Three possibilities existed for the mode of removal of vanadium from sea water by the ascidians. The first type would be an erratic assimilation in which no consistent variation of the remaining vanadium in the solution with time would be found. In the second case, the ascidians might concentrate constant amounts of vanadium per unit time, whereby a plot of the vanadium remaining in solution against time would give a linear relationship. Another possible mechanism would involve the ascidians concentrating the vanadium in direct proportion to the amount of vanadium present in the sea water environment. In such a case, a graph of the logarithm of the remaining amount of vanadium in the solution against time should result in a linear relationship. This last mechanism was found to be followed in experiments with *Ascidia ceratodes,* as is illustrated in Figures 1 and 2 where the logarithm of the vanadium remaining in solution (corrected for radioactive decay) is plotted against the time of withdrawal of the aliquot of sea water. In

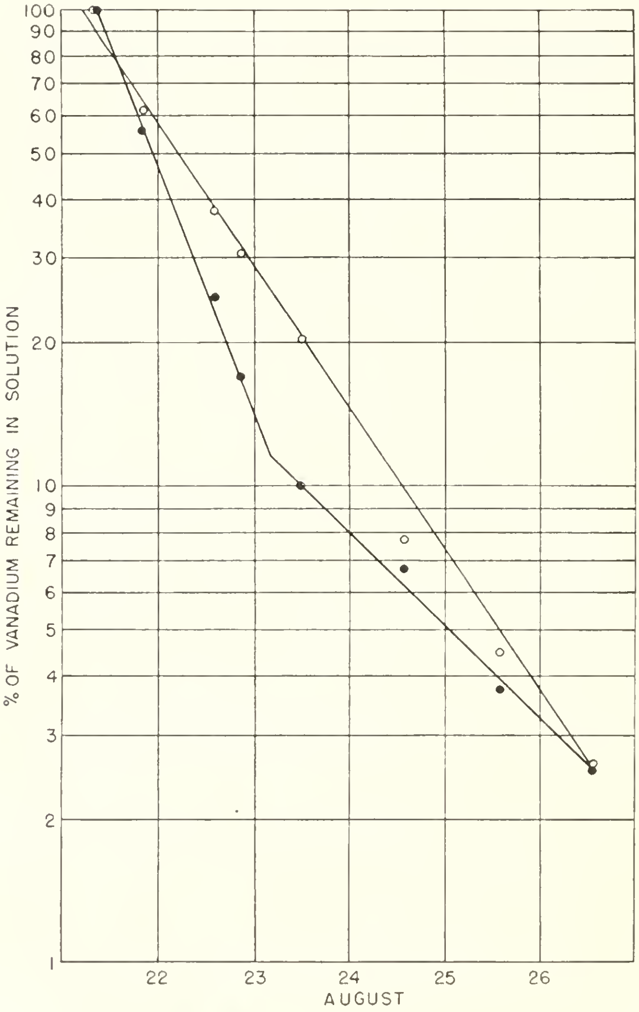

FIGURE 1. Two uptake curves of vanadium by *Ascidia ceratodes.* ○ Experiment 101. ● Experiment 2.

studies on *Ciona intestinalis* all of the curves obtained could be divided into two straight lines wherein a high initial uptake was followed by an abrupt change to a lower feeding rate. This is illustrated in Figure 2. In 10 experiments on *Ascidia ceratodes,* only two sharp changes were found in each case.

The instantaneous uptake of vanadium by these ascidians may be designated by

$$dN/dt = -VR \tag{2}$$

where V is the concentration of vanadium at time t in the sea water, N is the total amount of vanadium in the volume A of the sea water, and R is the effective clearance rate of the vanadium from the solution in units of volume per unit time,

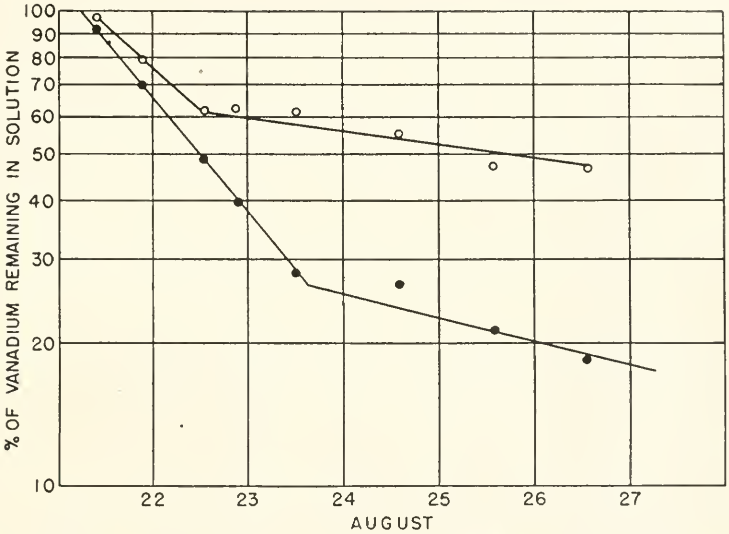

FIGURE 2. Two uptake curves of vanadium by *Ciona intestinalis.* ○ Experiment 7. ● Experiment 5.

assuming a 100 per cent uptake of vanadium. It follows that

$$V = N/A. \tag{3}$$

Utilizing Equation (3) and integrating Equation (2) from the beginning of the experiment we arrive at

$$V/V_0 = e^{-(R/A)t} \tag{4}$$

where V_0 represents the initial concentration of vanadium. For the purpose of comparative study we define the "half-time of vanadium in solution" as the time required for a given animal to reduce the vanadium concentration to half of its initial value. The values for the half-time for the 14 experiments conducted are listed in Table IV with the details of each run. Where changes occurred, the initial half-time is given.

The half-time will be a function of the effective adsorbing surface (*i.e.,* the geometry) and the rate at which the water is circulated through the animal. Where there is a change in the half-time during the course of an experiment, either or both of these parameters may change.

These experiments indicate that the initial transfer of vanadium from the hydrosphere to ascidians occurs by an adsorption process where the adsorbing surface is in an unsaturated state with respect to the adsorption of vanadium at all times, or is maintained in such a state by a constant regeneration of the surface. In either case the number of vanadium ions (or colloidal particles containing vanadium) would determine the number adsorbed at any given time.

To study more closely the dynamics of the vanadium transfer, the sea water in six experiments was enriched in phosphate ion. In the case of *Ascidia ceratodes* a high phosphate level inhibited the uptake of vanadium. This is seen in Experiments 4 and 104 where 50 microgram atoms of phosphate per liter were added initially to the sea water. In these two experiments the greatest half-times were observed. Five microgram atoms of phosphate per liter had little effect upon the uptake as is noted in Experiments 3 and 103. Since pentavalent vanadium ion can resemble phosphate ion chemically in solution, these experiments indicate that the phosphate ion competed successfully with the vanadium in the uptake process, and that vanadium in an ionic form rather than a colloidal vanadium is adsorbed. However, the solution chemistry of elements in concentrations in the level of vanadium in the sea and in our experiments has not been developed to the extent to give an unqualified answer.

TABLE IV

Half-times of vanadium in solutions containing ascidia

Experiment	Animal	Weight in Grams	Half-time Hours	Filter Treatment	Remarks
1	*A. ceratodes*	20.5 10.0	10	None	Two animals
2	*A. ceratodes*	13.5	14	Whatman 42	
3	*A. ceratodes*	23.0	24	Whatman 42	5μgr. at. $PO_4^{\equiv}$/liter
4	*A. ceratodes*	23.2	65	Whatman 42	50μgr. at. $PO_4^{\equiv}$/liter
101	*A. ceratodes*	29.8	24	None	
102	*A. ceratodes*	38.7	12	Whatman 42	
103	*A. ceratodes*	36.8	6	Whatman 42	5μgr. at. $PO_4^{\equiv}$/liter
104	*A. ceratodes*	30.9	48	Whatman 42	50μgr. at. $PO_4^{\equiv}$/liter
9	*A. ceratodes*	35.0	14	Silica column	
10	*A. ceratodes*	27.1	37	Silica column	
5	*C. intestinalis*	19.6	29	None	
6	*C. intestinalis*	25.4	22	Whatman 42	
7	*C. intestinalis*	25.6	40	Whatman 42	5μgr. at. $PO_4^{\equiv}$/liter
8	*C. intestinalis*	47.6	18	Whatman 42	50μgr. at. $PO_4^{\equiv}$/liter

PUMPING RATE STUDIES ON CIONA INTESTINALIS

To determine the efficiency of transfer of the vanadium from the sea water to the animals, investigations concerning the effective pumping rates of *Ciona intestinalis* were conducted. In each experiment a single animal was placed in a small plastic aquarium two inches deep (Fig. 3). A continuous flow of sea water

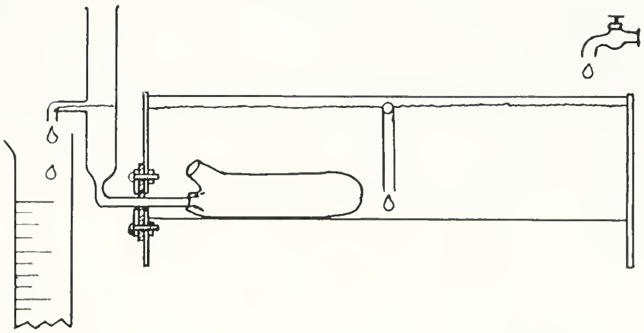

FIGURE 3. Aquarium used for pumping rate studies of ascidians.

was maintained and the water level was kept constant by means of drains placed at the top of the aquarium. A Pyrex glass tube of 4 mm. outer diameter with a 6 mm. outer diameter bulb at one end was inserted in the opened exhale syphon of the ascidian with great care to avoid injury to the animal. The animals soon relaxed with such an arrangement. The ascidian, with inserted tube, was then placed in the aquarium and the tube connected to a second glass tube which entered the aquarium through a rubber gasket and discharged the water outside of the aquarium at the same level as the drains. There was therefore no hydrostatic head to aid

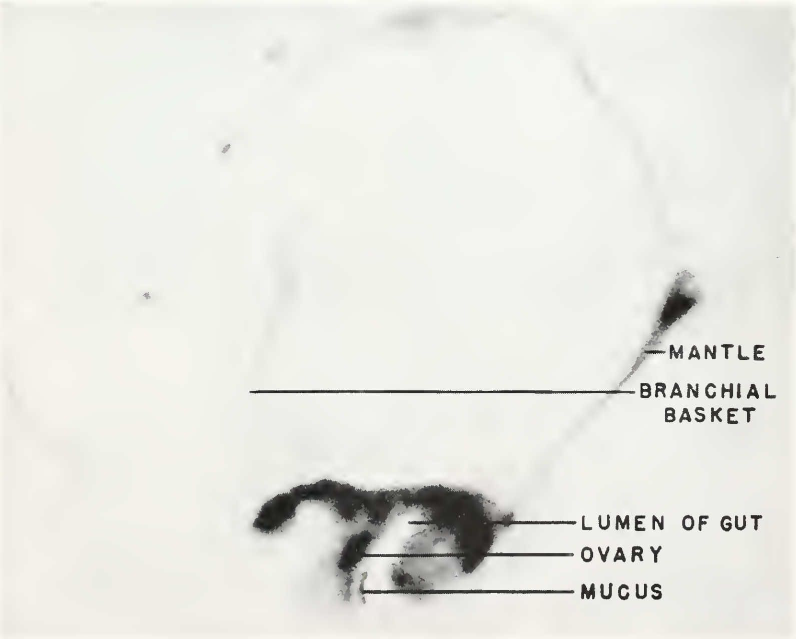

FIGURE 4. Radioautograph of *Ascidia ceratodes* used in Experiment 9. Concentration of vanadium is seen in ovary, gut wall, and in the mucus in the gut. Fifteen day exposure.

or hinder the ascidian in its normal pumping functions. The animals pumped at a constant rate after the tube had been inserted for two hours. The animals seemed to seal their junction to the tube by means of mucus and muscular tonus.

For an ascidian with a wet weight of 25 grams, pumping rates of about two liters per hour were observed. This result compares favorably with the pumping studies of Jørgensen (1949) on *Ciona intestinalis*; using a different technique, he obtained values of the same order of magnitude.

RADIOAUTOGRAPHIC STUDIES

At the completion of the uptake studies with radio-vanadium the animals were sacrificed for radioautographic analysis. Both *Ascidia ceratodes* and *Ciona intestinalis* were fixed in Bouin's solution, imbedded in paraffin, and sectioned at about 100 micra. Survey autographs were obtained with Eastman No-Screen X-Ray film with exposure times up to 30 days. Figures 4 and 5 show two typical transverse sections.

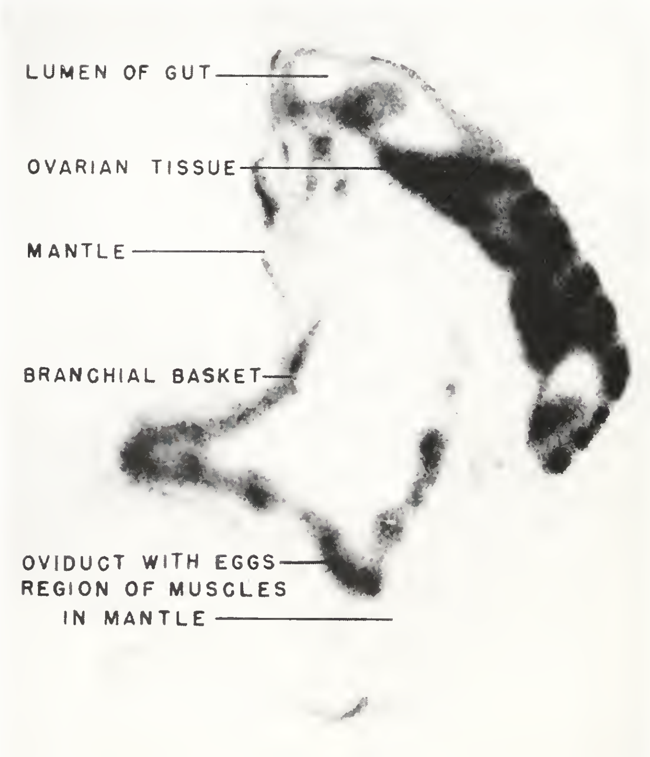

FIGURE 5. Radioautograph of *Ascidia ceratodes* used in Experiment 102. Concentration of vanadium is seen primarily in ovary, gut wall, oviduct (eggs), and branchial basket, with very small amounts in the mantle. No vanadium concentration was found in the muscles of the mantle. Exposure time of 15 days.

The autographs confirmed the chemical analyses on *Ciona intestinalis,* showing high concentrations in the gut wall and in the ovary, and showed similar local concentrations in *Ascidia ceratodes.* The vanadium concentration in the ovaries of both species is primarily in the follicles. The vanadium was still in the mature eggs when they were extruded from the ovary into the peribranchial cavity.

There is some concentration of the vanadium in the branchial basket of both species, though sometimes not in very great amounts. This enrichment may be due to the blood vessels in this structure, as high concentrations of vanadium in blood vessels were observed. The mantle and test showed very little vanadium and the muscles of the mantle displayed no evidence of this element. Vanadium appeared in the mucus of the branchial basket and in an isolated bit of mucus in the gut as is shown in Figure 4. The absence of high amounts of vanadium in the branchial region may have been due to washing away of the mucus sheet during the fixing procedure or to the constant renewal of fresh mucus by the living animal.

Discussion

The uptake experiments establish that vanadium can be concentrated directly from solution by the two species of ascidians used in this work. To obtain an idea of the efficiency of this adsorption process, we may compare the effective clearance rate of vanadium with the pumping rates for *Ciona intestinalis.* By substitution in Equation (4) of $A = 2$ liters/hour, $V/V_0 = \frac{1}{2}$, and the half time = 30 hours (the average from Experiments 5, 6, and 7), we arrive at an effective clearance rate of vanadium of 0.05 liters per hour. Compared with the observed pumping rate of 2 liters an hour, the efficiency of uptake of vanadium is of the order of 2½ per cent. This is the value for animals which were unfed, since food in the form of particulate matter could not be introduced without adsorbing vanadium from solution and invalidating the results.

The extraordinary biological specificity of the adsorbing surface of the ascidians for the vanadium in aqueous solution remains an unsolved problem of high biochemical interest. This selectivity is undoubtedly related to the unique ionic structure and properties of vanadium. The mucus sheet fulfills the requirement of a constantly renewed surface. The high vanadium content of the mucus, as shown by the autographs, in the branchial basket and in the gut, points to this sheet as the adsorbing surface. The sharp discontinuities in the uptake curves may be explained by chemical or area changes in the adsorbing surface.

The high concentrations of vanadium in the ovarian follicles, as shown in the autographs, suggest that this element may become part of a metabolic system that is present from the beginning in the developing embryo. Further work utilizing more concentrated radioactive vanadium solutions and thinner autograph specimens should reveal more on the intimate histology of vanadium in these animals.

We may now attempt to account for the high amounts of vanadium found in ascidians. In the case of *Ciona intestinalis* which contains about 100 micrograms of vanadium in a mature 20 gram animal, a minimum of 450 liters of sea water may be effectively cleared of vanadium in a year. This is equivalent to about 45 micrograms of vanadium. Further, we must consider that vanadium and many other elements are ionically or colloidally adsorbed on the particulate matter of the oceans. This phenomenon presented itself in the preliminary feeding experiments.

Thus, a more concentrated source of vanadium is available to these filter feeders. A final potential store of vanadium lies in the plankton of the oceans, a source of ultimate food for these filter feeding animals.

SUMMARY

1. Chemical analyses made on *Ciona intestinalis* indicated the vanadium to be localized in the gut region and the ovaries. These sites of assimilation were confirmed by radioautographs utilizing cyclotron-produced radioactive vanadium.

2. The total vanadium content of four local species of ascidians was determined. The uptake of radioactive vanadium from sea water indicated that *Ciona intestinalis* and *Ascidia ceratodes* are able, by means of an adsorption mechanism, to concentrate vanadium directly from sea water. The efficiency of assimilation was of the order of $2\frac{1}{2}$ per cent for unfed animals.

3. Pumping rate of water by *Ciona intestinalis* was determined by a direct method. It was concluded that sea water and its particulate constituents could furnish the necessary vanadium demanded by *Ciona intestinalis*.

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RELATIONSHIP BETWEEN NUCLEAR VOLUMES, AMOUNT OF INTRANUCLEAR PROTEINS AND DESOXYRIBOSENUCLEIC ACID (DNA) IN VARIOUS RAT CELLS

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The concept that increase in size of nuclei and cells within one tissue is directly correlated with an increase in chromosome number or chromosomal mass has become more or less accepted in cytology (Hertwig, 1934, 1939).

But Montgomery (1910), Bowen (1922) and Schrader (1947) have shown that in different sizes of spermatocytes in pentatomid testes, the chromosome number and chromosome size are identical in the different sized cells. Recently, Schrader and Leuchtenberger (1950), using quantitative cytochemical methods, presented evidence that in the pentatomid Arvelius there exists a direct correlation between three sizes of nuclear volumes of spermatocytes (200, 400, 1600 cubic microns), and the amount of contained proteins, while the amount of DNA was the same in these nuclei. On the basis of these results, the assumption was made that changes in nuclear sizes in a tissue are due mainly to alterations in protein and water content, and not due to different amounts of DNA.

The present communication presents further evidence on a parallelism between size of nuclei and amount of intranuclear protein within one tissue, but points out that such a correlation does not apply to a tissue containing cells with different synthetic activities, such as the parietal and chief cells of the gastric mucosa.

MATERIAL AND METHODS

Rat tissue was fixed immediately after death in 50 per cent formalin and Carnoy and cut at different thicknesses, according to the requirements of the investigation. The estimation of DNA and protein was done by the cytochemical and photometric microscopic methods previously described (Schrader and Leuchtenberger, 1950; Pollister, 1950).

RESULTS

As can be seen from the data in Table I, there occur three distinct classes of DNA, namely 3.3, 6.6 and 12.8, in liver nuclei of the rat, an observation which is in agreement with the findings of Ris and Mirsky (1949) and Leuchtenberger, Vendrely and Vendrely (1951). But it is also evident that in contrast to the nearly exact multiples of DNA in the nuclei (1, 2, 4), the nuclear volumes do not show such a multiple relationship, but vary over a large range with intermediate values. It can be furthermore seen that there is no correlation between nuclear size and amount of DNA per nucleus; within each DNA class the size of nuclei varies considerably. For instance, among 36 nuclei with a DNA content of 6.6 per nucleus,

TABLE I

Relationship between nuclear volumes, amounts of intranuclear proteins and desoxyribosenucleic acid of rat liver cells*

	Mean Amount of DNA per Nucleus = 3.3 ± 0.08			Mean Amount of DNA per Nucleus = 6.6 ± 0.24			Mean Amount of DNA per Nucleus = 12.8 ± 0.50		
Mean nuclear volumes in cubic microns	62.0	153±17	289±27	285±20	455±30	732±78	376	637	1138
Number of nuclei measured	3	20	6	18	13	5	4	4	6
Mean amount of protein per nucleus	1.9	4.6±0.23	8.7±0.3	8.6±0.1	13.7±0.5	22.0±1.2	11.3	19.0	34±1.4

* Amounts expressed in arbitrary units.

18 showed a nuclear volume half or less than that of the remaining nuclei. The independence of nuclear size and of DNA content is also obvious if one compares the nuclear volumes of one DNA class with those of another DNA class; *i.e.*, 6.6 DNA per nucleus occurs in cells with nuclear volumes which are as small as those of the 3.3 and as large as those of the 12.8 DNA class. The occurrence of nuclear volumes in nonmultiples is in agreement with the data of Biesele (1944) on volumes of rat liver nuclei.

The picture is considerably different if one considers the amount of protein per nucleus in relation to nuclear size. It is evident that there exists a direct correlation between both; any increase in nuclear volume is accompanied by a corresponding increase in amount of intranuclear protein. In order to see whether such a close relationship between size of nuclei and amounts of contained proteins would hold in tissues in which cells with different functions occur, we examined the gastric mucosa of the rat. It is known that there the chief cells produce a protein, namely a precursor of pepsin, while the parietal cells produce HCl. In the following Table II, the results are presented.

On the basis of the data in Table II it is evident that the amount of DNA per nucleus is the same in chief cells and parietal cells. It can be further seen that

TABLE II

Relationship between nuclear volumes, amounts of intranuclear proteins and desoxyribosenucleic acid of rat gastric mucosa cells*

Type of Cell	No. of Nuclei Measured	Nuclear Vol. in Cubic Microns	DNA per Nucleus	Mean amount of Protein per Nucleus
Parietal Cell	19	57±4	2.9±0.1	1.0±0.1
Chief Cell	20	44±2	3.1±0.1	1.9±0.09

* Amounts expressed in arbitrary units.

though the nuclear volumes of the parietal cells are larger than those of the chief cells, the latter contain a larger amount of protein per nucleus than the parietal cells. Therefore, the strict parallelism between amounts of proteins and nuclear size, as found in liver cells or spermatocytes, does not necessarily hold for cells which differ in the nature of their synthesizing activities, such as the chief and parietal cells of the gastric mucosa.

Discussion

The close correlation between amount of intranuclear proteins and nuclear volumes, and the independence of the amount of DNA from the latter in rat liver nuclei is in good agreement with the previous work of Schrader and Leuchtenberger (1950) concerned with proteins and DNA in spermatocytes.

A similar correlation between amount of proteins and nuclear sizes in oocytes of mice was recently reported by Alfert (1950). The striking parallelism between amounts of proteins and size of nuclei in all these tissues is based on the fact that the concentration of proteins stays the same in all nuclei, whether the cell decreases or increases in size.

But these observations do not apply to all the tissues of the same animal, as can readily be seen from the results in cells of the gastric mucosa. It seems that the correlation exists only if cells actually have the same metabolic function as, for instance, the cells of the parenchyma of liver or the spermatocytes in the testes. However in a tissue in which cells occur having different synthetic activities, the concentration of the proteins may vary. In the gastric mucosa, the protein-producing chief cells show nearly twice the amount of protein per nucleus, though their nuclear volume is smaller than that of the HCl-producing parietal cells. These results are in good agreement with Caspersson's work (1950), who, on the basis of ultraviolet studies, reported considerably larger amounts of proteins and ribosenucleic acid in chief cells than in the parietal cells.

Whether it can be assumed generally that variable concentrations of proteins in different cells of the same tissue indicate differences in protein synthesis cannot be decided at this time. But the possibility is now opened that the cytochemical photometric determination of proteins in individual cells may allow such a differentiation.

A comparative study of the protein content of nuclei of normal and abnormal cells is now in progress.

Summary

Cytochemical photometric microscopic determinations of proteins and desoxyribosenucleic acid in cells of rat tissues gave the following results:

1. A direct correlation exists between different sizes of nuclei and amounts of intranuclear proteins in rat liver cells, while the amount of DNA was found to be independent of nuclear size.

2. In the gastric mucosa, where cells with different synthetic activities (parietal and chief cells) occur, there is no parallelism between nuclear size and proteins. The chief cells have a larger amount of protein than parietal cells, while the amount of DNA is the same in both.

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ACTION OF VARIOUS ENZYMES ON THE MATING TYPE SUBSTANCES OF PARAMECIUM CALKINSI[1]

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When paramecia of complementary mating type are mixed under appropriate conditions, these animals adhere to one another and shortly form large clumps or agglutinates. All available evidence (Kimball, 1943; Metz, 1948) indicates that this initial agglutinative mating reaction results from interaction of complementary substances or at least complementary configurations on the surfaces of the cilia. For convenience these substances or configurations are called the mating type substances.

Recent studies have shown that interaction of mating type substances may perform a far more significant function in fertilization than the obvious one of effecting the initial union of potential conjugants. Thus, Metz and Foley (1949) using a mutant stock of *Paramecium aurelia* found that only the initial mating reaction union is essential for induction of meiosis, macronuclear breakdown, loss of mating reactivity and pseudo selfing pair formation in this species. Since the mating reaction alone was essential for induction of these activation phenomena, it seemed reasonable to conclude that interaction of mating type substances initiates the activation. In view of this, it is of interest to obtain more specific information regarding the physical basis for the mating reaction, in other words to define the mating type substances in more concrete physico-chemical terms. In an attempt to achieve this goal an investigation of the chemical nature of the mating type substances has been undertaken. As one phase of this study several enzymes were tested for action on the mating activity of paramecium. *Paramecium calkinsi* was selected for this study because of the ease with which cultures of this species may be obtained in mating condition. Of the several enzymes tested, only the proteolytic enzymes, trypsin and chymotrypsin, affected the mating reactivity of the paramecia.

MATERIAL AND METHODS

Enzyme preparations. The following enzyme preparations were tested for their effects on the mating reactivity of *P. calkinsi*: crystalline preparations (Armour) of trypsin, chymotrypsin, lysozyme and ribonuclease; purified hyaluronidase and crude preparations of ptyalin and lecithinase (bee venom, prepared by the method of Flury, 1920).

Preparation of the paramecia for testing. As pointed out elsewhere (Metz and Butterfield, 1950), paramecia in mating condition quickly lose their mating reactivity when fed bacteria. Furthermore, paramecia appear to feed upon concentrated protein solutions (enzymes, rabbit sera) since they enlarge, become filled with food vacuoles and sometimes divide in such solutions. To avoid the possibility of such

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indirect loss of mating activity from feeding, the paramecia were killed with formalin or picric acid before treatment with enzyme. Paramecia killed by appropriate treatment with these agents give strong, specific mating reactions. Indeed, such dead animals can activate living animals of opposite type (Metz, 1948). The reader is referred to the article of Metz and Butterfield (1950) for the methods of culturing and killing the *P. calkinsi*.

Method of treatment with enzyme. To test for action on the mating substance, both the enzyme preparation and the formalin- or picric acid-killed reactive animals were taken up in an appropriate buffer with pH adjusted near the optimum for enzyme action. Equal volumes of enzyme solution and paramecium suspension were then mixed in depression slides or precipitin tubes. After an appropriate incubation period (1–6 hrs., 20°–30° C.) the treated animals were washed free of enzyme solution with ⅖ sea water and tested for mating reactivity with living reactive type I and II animals. To control for reactivity of the dead animals and to check for inactivation of the animals by the buffer, equivalent samples of the same dead paramecia were treated with buffer but without enzyme solution. When appropriate known enzyme substrates were available, these were employed as controls for enzyme activity. To accomplish this, samples of the enzyme solution and a solution of appropriate known substrate in the same buffer were mixed and incubated in parallel with the treated paramecia. In a few cases the experiment was set up in such a manner that the enzyme digested an amount of known substrate equal to or greater than the mass of the enzyme treated paramecia. The mass of the paramecia was determined roughly by counting the animals present and multiplying by 0.05, the weight (in micrograms) of one *Paramecium calkinsi*. This figure was obtained from the number (7.5×10^5) of paramecia in a 37.3 mg. sample of washed, lyophylized animals. It agrees reasonably well with the figures of Boell and Woodruff (1941).

As a final control, the supernatant buffer from a sample of the dead paramecia usually was tested for inhibition of enzyme action. Conceivably substances leaching from the dead animals could inhibit the enzyme in the test sample. Traces of the formalin or picric acid used to kill the paramecia would fall into this category. In all cases where this control was employed, the enzyme was active in the animal supernatants.

RESULTS

Non-proteolytic enzymes. Crystalline (Armour) preparations of lysozyme and ribonuclease, purified (1300 turbidity reducing units/mg.) hyaluronidase and crude preparations of ptyalin and lecithinase (bee venom) all had no detectable effect upon the mating reactivity of dead *P. calkinsi*. The conditions under which each of these enzymes was tested are given in Table I.

Proteolytic enzymes. In marked contrast to the preparations considered above, the proteolytic enzymes trypsin and chymotrypsin destroyed the mating reactivity of formalin- or picric acid-killed *Paramecium calkinsi* of both mating types. Five-tenths per cent crystalline (Armour) trypsin and chymotrypsin solutions were made up in borate buffer, pH 8. Equal volumes of these enzyme solutions and suspensions of dead paramecia in the same buffer were mixed and allowed to stand. At intervals samples of dead animals were removed from the enzyme solution, washed in ⅖ sea water and tested for mating reactivity with living type I and II test animals. A portion of each sample was also stained with iodine solution and examined under the oil immersion objective for the condition of the cilia. Dead animals from the same original sample but treated with buffer only (no enzyme) served as the standard of comparison both for mating reactivity and condition of the cilia. The results of a typical experiment with trypsin and chymotrypsin are given in Table II. It is evident from the data of Table II that the trypsin and chymotrypsin preparations destroy the mating reactivity of both type I and II animals.

The striking difference in time for inactivation of formalin-killed as opposed to picric acid-killed animals is not readily explained. Possibly this difference in inactivation time simply reflects a difference in the number of reactive groups exposed on the surfaces of the paramecia. This view is supported by the fact that formalin-killed animals usually give stronger mating reactions than picric acid-killed animals. Possibly traces of formalin leaching from the dead animals inhibit the enzymes to a greater degree than corresponding traces of picric acid. Finally, differences in the extent of alteration ("denaturation") of paramecium proteins by formalin, as opposed to picric acid, may account for these results. In this connection it will be recalled that denatured proteins are known to be more susceptible to the action of proteolytic enzymes than the native material (Haurowitz, 1950).

Table I

Conditions under which non-proteolytic enzymes were tested for action on formalin- and picric acid-killed Paramecium calkinsi

Enzyme	Animals Treated*	Buffer	pH	Final Enzyme Concentration	Temperature	Digestion Time	Inhibition of Enzyme by Supernate	Mg. Animals Treated with Enzyme	Mg. Known Substrate Digested
Bee venom	F I	2.5 sea water	6.9	0.63%	28° C.	1.5 hrs.	No inhibition	0.045	Hemolyzed 2% RBC suspension
Hyaluronidase[2]	F I	2.5 sea water	6.9	0.63%	28° C.	2 hrs.		0.018	
Lysozyme	F I	0.0025 N NaCl	6.6	0.5%	Room temp.	2.7 hrs.	No inhibition	>1.0	Lysis of turbid *S. lutea* suspension
	P I	NaCl	6.6	0.5%	Room temp.	2.6 hrs.		>1.0	
	P II	NaCl	6.6	0.5%	25° C.	2.0 hrs.		>1.0	
Ptyalin	F I, P II	Phosphate	6.6	1/2 sat. sol.	37° C.	2.0 hrs.	No inhibition	0.4	0.6 mg. soluble starch
Ribonuclease[3]	F I	Borate	8.0	0.075%	30° C.	6 hrs.	No inhibition	0.1 mg. paramecium nitrogen	0.7 mg. Na nucleate nitrogen
	P I, P II	Borate	8.0	0.05%	Room temp.	6 hrs.			>5 mg. Na nucleate

* F I indicates formalin-killed type I animals; P I, picric acid-killed type I animals; and P II, picric acid-killed type II paramecia.

[2] The authors are indebted to Dr. J. Seifter, Wyeth Institute of Applied Biochemistry, for this preparation of hyaluronidase.

[3] The ribonuclease preparation was found to hydrolyze casein. This proteolytic action was removed by the method of McDonald (1948).

As might be expected, the enzyme preparations not only destroy the mating reactivity of the dead animals but in time they digest the cilia and eventually reduce the formalin- or picric acid-killed animals to fragile ghosts. However, the enzyme-treated dead animals can lose their ability to give the mating reaction before the cilia show any appreciable action of the enzyme. Cilia of control formalin- or picric acid-killed paramecia are usually straight and extend in a radial direction from the body of the animal. After treatment with trypsin or chymotrypsin, however, the cilia (iodine stained) gradually lose sharpness of outline, become curly and finally disappear. The picric acid-killed animals of Table II still possessed cilia after a 17 minute treatment with trypsin. However, the cilia of these animals could be distinguished from those of the controls. The former were classified as "fair to good" (picric acid-killed type I animals at 17 minutes) or "poor to fair" (picric acid-killed type II animals) when compared for sharpness of outline and straightness with the controls. Likewise, picric acid-killed, chymotrypsin-treated animals retained their cilia for some time following loss of mating activity. Even at 70 minutes these type I animals (Table II) possessed fair to good cilia. The cilia of some animals were indistinguishable from those of controls. At 165 minutes, however, these animals were devoid of cilia. The picric acid-killed type II animals had "poor to fair" cilia after 60 minutes treatment with chymotrypsin and no cilia after 165 minutes treatment.

TABLE II

Mating reactivity of trypsin- and chymotrypsin-treated dead Paramecium calkinsi

Enzyme	Digestion Time	Enzyme Treated			Buffer Treated		
		Formalin-killed I	Picric Acid-killed		Formalin-killed I	Picric Acid-killed	
			I	II		I	II
Trypsin	17 min.	++++	—	—	++++	++-+++	+++
	62 min.	++	—	—	++++	++	++
	137 min.	++	—	—	++++	++	++-+++
Chymotrypsin	25 min.	+++	—	+	++++	++	+++
	70 min.	++	—	—	++++	++	+++
	165 min.	++	—	—	++++	++	++

From these and other observations on trypsin and chymotrypsin treated animals, it is evident that loss of mating activity does not necessarily involve any serious visible change in the structure of the cilium. Thus, the loss of mating reactivity effected by these enzymes results initially from an alteration in the surface structure of these organelles. Interpretation of this effect in terms of a specific protein substrate presents certain difficulties, however. The proteolytic enzymes could act directly on the reactive groups (*i.e.,* the "mating substance"), in which case these reactive groups would be proteinaceous. On the other hand, the enzymes might destroy mating reactivity indirectly by breaking down protein in the superficial layers of the cilium. Such action might alter the relations (*i.e.,* spacing) of essential non-protein surface groups or even release the essential material into solution. This latter possibility was tested as outlined in the following section.

Failure of trypsin digests of reactive paramecia to act on animals of opposite type. Reactive *P. calkinsi* type I in four liters of culture were treated with formalin. The resulting reactive dead animals were washed in saline and pH 8 phosphate buffer. The final yield of 0.7 ml. of packed dead animals was taken up in one ml. of the buffer. Five-tenths ml. of this sample was mixed with an equal volume of 0.5 per cent trypsin in the same buffer. The remaining 0.5 ml. of suspension (control) was treated with buffer alone. The two suspensions were incubated at 32° C. for 5 hours. At the termination of this digestion period the enzyme-treated animals were reduced to fragile ghosts which gave no mating reactions. Animals of the control sample (treated with buffer alone) appeared to be in a good state of preservation and gave good mating reactions. The unreactive ghosts were now centrifuged from the trypsin-treated suspension and 0.25 ml. of one per cent trypsin inhibitor (ovomucoid prepared as described by Lineweaver and Murray, 1947) was added to prevent action of the enzyme on test animals. This trypsin inhibitor-treated, trypsin digest of formalin-killed *P. calkinsi* type I was tested for its ability selectively to destroy mating reactivity of type II animals. Eight drop samples of the digest were mixed with three drop samples from suspensions of diluted, freshly prepared, picric acid-killed reactive type I and II animals. One hour later these dead animals were washed in saline and tested for mating reactivity. The digest-treated type I and II animals both gave mating reactions of intensity equal to that of buffer-treated controls. Evidently, then, the trypsin digest of reactive type I animals has no mating reaction inhibiting properties.

Discussion

None of the several non-proteolytic enzymes employed had any detectable effect upon the mating reactivity of the paramecia. Assuming that these experiments were critical, this result indicates that the natural substrates of these enzymes are not essential constituents of the mating type substances of *P. calkinsi.* Elimination of these classes of substances as possibilities is not without interest since certain of them are known to be present in intercellular cement (hyaluronic acid) or cell membranes and cell surfaces (bacterial mucopolysaccharides hydrolyzed by lysozyme, Meyer and Hahnel, 1946; ribonucleic acid in Arbacia egg surface, Lansing and Rosenthal, 1949; lipoprotein attacked by lecithinase in erythrocyte and sea urchin egg membranes, Runnström, Tiselius and Lindvall, 1945).

As suggested elsewhere in this article the inactivating action of trypsin and chymotrypsin can be interpreted either as a direct effect upon the essential groups of the mating substance or an indirect action upon neighboring protein. Since no mating substance activity could be demonstrated either from the animals or the supernatant fluid following treatment with these enzymes, it appears that some essential molecular structure was destroyed. This suggests that the essential groups are protein or that they are intimately associated with protein. Insofar as the presence of immunizing antigens indicates protein, the immunological studies of Metz and Fusco (1948) support this view. It is of interest in this connection that the latter study indicates that the mating substance is associated with antigenic material but that the essential groups are not themselves antigenic.

Unfortunately, no more positive statement regarding the chemical nature of the mating substance seems warranted on the basis of the information presented here.

If mating substance activity is dependent upon protein integrity, as seems to be the case, then the protein material must be of rather unusual character. Its activity is not destroyed by fairly harsh treatment with certain strong protein-denaturing agents such as formalin, picric acid and mercuric chloride. Furthermore, the rate of inactivation of mating substance by trypsin and chymotrypsin may depend upon previous treatment (*i.e.*, formalin vs. picric acid). If true, this would also be an unusual situation since any substance or group of substances with as complex and highly specific action as these would be expected to show some differences in activity to correspond with differences in susceptibility to enzymes. Unfortunately no satisfactory method has been devised to assay mating substance activity. Therefore serious quantitative studies of the reactivity of formalin- as opposed to picric acid-killed animals, both before and after enzyme treatment, must await development of an appropriate technique. In view of these considerations, the possibility that the mating type substances of *P. calkinsi* are proteins is accepted as a working hypothesis with the customary reservations.

Aside from the question of the chemical nature of the mating type substances, the mating substance inactivating action of proteolytic enzymes is of interest in connection with the inhibiting action of certain paramecium extracts. Metz and Butterfield (1950) have shown that homogenates of *P. calkinsi* contain a heat-labile, non-type specific mating substance inhibiting agent. It was suggested that this agent might be an enzyme. Considering the present findings it seems possible that the agent in question is a proteolytic enzyme. Such an enzyme need not bear any physiological relation to the mating substances. Indeed, it may well be a proteolytic enzyme which is released into the food vacuoles in the living animals and which under normal conditions participates in the digestion of the contents of these vacuoles (see Mast, 1947). Upon homogenizing the paramecia, such an enzyme could be released into the extraction fluid and under the conditions of the experiments would be expected to inactivate the mating substances of the test animals.

SUMMARY

1. In an attempt to characterize the mating type substances of *Paramecium calkinsi*, several enzymes were tested for ability to destroy the reactivity of formalin- or picric acid-killed animals.

2. The following non-proteolytic enzymes had no detectable effect upon the mating reactivity of *P. calkinsi*: Lecithinase (bee venom), hyaluronidase, lysozyme, ptyalin, ribonuclease. Insofar as these experiments were critical, it is concluded that the natural substrates for these enzymes are not essential constituents of the mating type substance of *P. calkinsi*.

3. Crystalline preparations of the proteolytic enzymes trypsin and chymotrypsin destroyed the mating reactivity of both type I and type II animals. Mating substance activity was not found in the digest of enzyme-treated paramecium.

4. On the basis of these findings it is concluded that mating reactivity is dependent upon protein integrity. Possibilities regarding the nature of this dependence are discussed.

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NEUROSECRETION. X. A RELATIONSHIP BETWEEN THE PARAPHYSIS AND THE PARAVENTRICULAR NUCLEUS IN THE GARTER SNAKE (THAMNOPHIS SP.) [1]

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In an earlier paper in this journal (Scharrer and Scharrer, 1944) it was shown that in vertebrates the stainable material produced by the neurosecretory cells of the hypothalamus passes along the axons of these cells toward the hypophysis. Similar material elaborated by the neurosecretory cells of the pars intercerebralis in insects also "migrates" along nerve fibers toward an organ complex, namely the corpora cardiaca and allata, which corresponds in many ways to the hypophysis of the vertebrates. The concept of two analogous neuroendocrine systems in invertebrates and vertebrates, which was established on the basis of these observations, has proved fruitful. As to the intercerebralis-cardiacum-allatum system of the insects, Thomsen (1948) studied a possible relationship between the neurosecretory activity of the pars intercerebralis and the "gonadotropic" principle of the corpus allatum. The study showed a positive relationship in that extirpation of the pars intercerebralis in the fly, *Calliphora erythrocephala*, prevented the production or release of the gonad-stimulating principle of the corpus allatum and resulted in the inability of the ovary to produce mature eggs. Also, the concept of an analogous hypothalamo-hypophyseal system in vertebrates has been extended further by Bargmann (1949), Bargmann and Hild (1949), Hild (1950), Ortmann (1950), Bargmann, Hild, Ortmann and Schiebler (1950) and Smith (1951). There is now convincing evidence, recently reviewed by Bargmann and Scharrer (1951), that the stainable material produced by the secreting nerve cells of the hypothalamus and passing along their axons to the neurohypophysis is related to the hormones which can be extracted from the pars nervosa of the pituitary gland.

If this conclusion is correct, neither the corpus cardiacum of the insects nor the neurohypophysis of the vertebrates are endocrine organs in a strict sense, but serve principally as sites of storage of the products of neurosecretory cells in the brain.

Questions arise as to whether (a) the neurohypophysis and the corpus cardiacum are the only organs into which the secreting cells of the hypothalamus and the pars intercerebralis respectively discharge their products, and (b) neurosecretory centers other than those in the hypothalamus of the vertebrates and the pars intercerebralis of the insects are also connected with reservoirs which receive the products of neurosecretory activity by way of axons and store them for release according to need. Both problems are at present under investigation. Observations reported in this paper concern a relationship between the neurosecretory cells of the paraventricular nucleus and an organ other than the pars nervosa of the pituitary, namely the paraphysis, in the garter snake.

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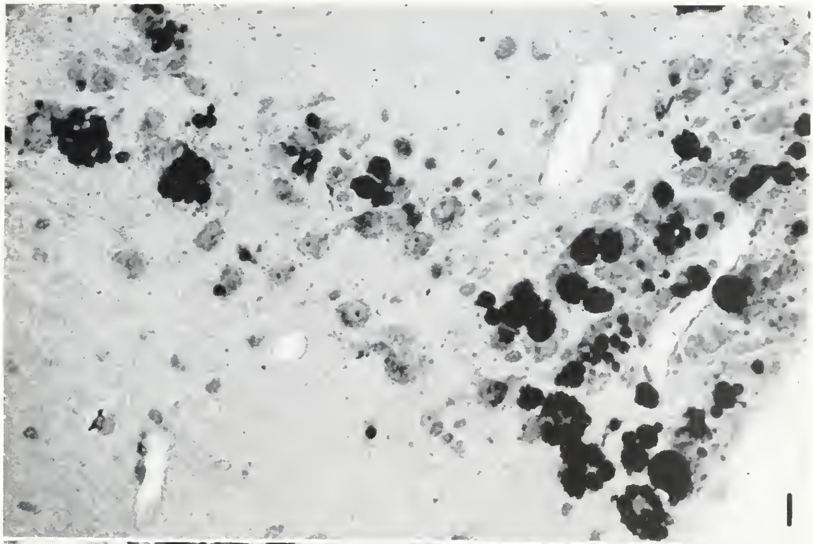
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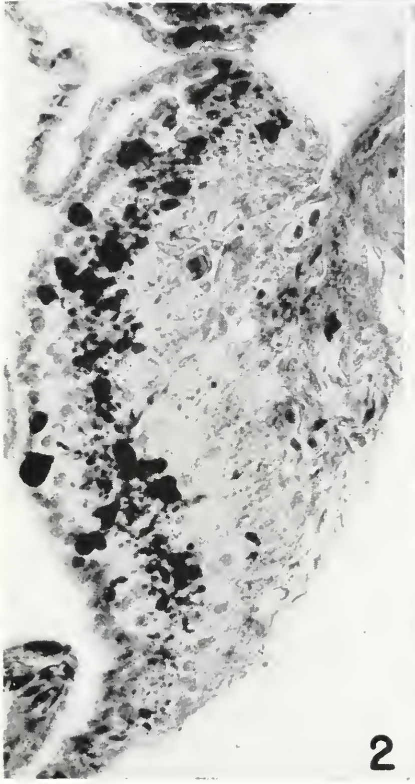
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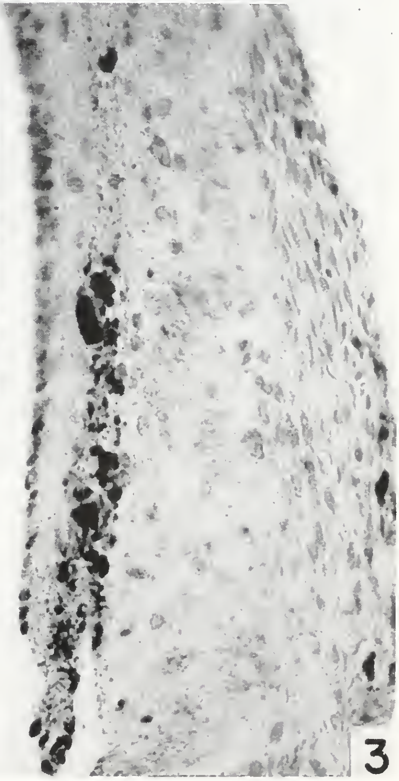
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FIGURES 1 to 7: Thamnophis sp., 5 micra sections; Gomori's chrome alum hematoxylin phloxine; photomicrographs.

FIGURE 1. Nucleus supraopticus. Secretory granules and droplets are stained deep blue. × 440.

FIGURE 2. Cross section of stalk of pituitary with stainable material from neurosecretory cells in the hypothalamus. × 400.

FIGURE 3. Longitudinal section through stalk of pituitary with stainable material from neurosecretory cells in the hypothalamus. × 400.

MATERIALS AND METHODS

The brains of garter snakes (Thamnophis sp.) were fixed with Zenker-formol by immersion or perfusion through the heart. They were embedded in paraffin; sections of 5 micra thickness were stained with chrome alum hematoxylin phloxine as described by Gomori (1941).

OBSERVATIONS

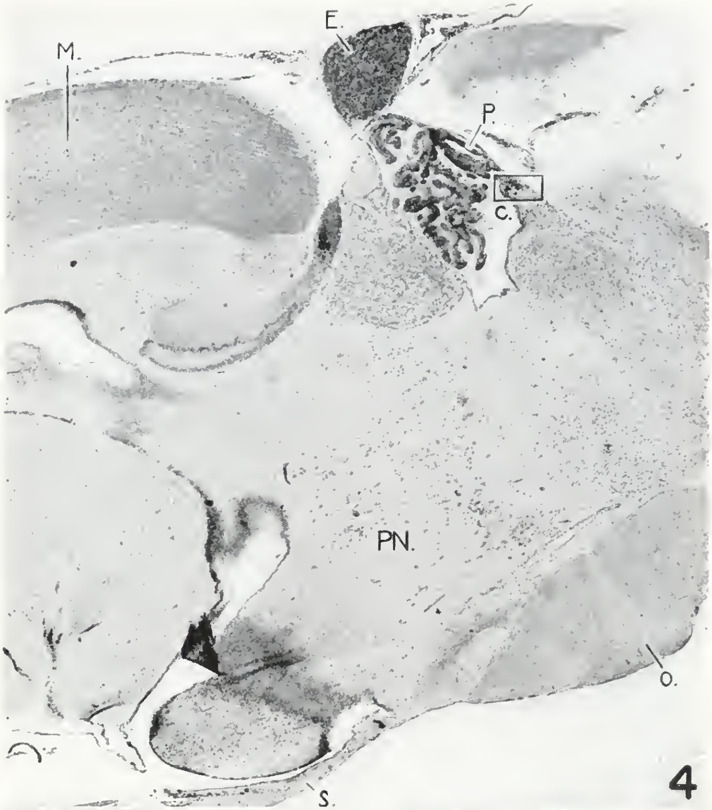
In snakes, as in higher vertebrates, there are two nuclei in the hypothalamus whose cells show secretory activity: the nucleus supraopticus and the nucleus paraventricularis (Scharrer, 1933). The location of these nuclei in the garter snake is illustrated in Figure 8.

The cells of the supraoptic and paraventricular nuclei are distinct in that they contain many fine granules and droplets of varying size. This material stains dark blue with Gomori's chrome alum hematoxylin phloxine method (Fig. 1). The elaboration of this stainable material in granular form is characteristic of the secretory activity of these cells, not only in the snakes but in the homologous nuclei of lower as well as higher vertebrates.

These granules, as in the case of other animals, are contained in the processes of the cells which can, therefore, be traced with great accuracy. It was shown in an earlier paper of this series (Palay, 1945) that in fishes the axons provide a means by which the granules are removed from the neurosecretory cells. This "neurosecretory pathway" in snakes, as in other vertebrates, leads to the pars nervosa of the pituitary gland (Bargmann, Hild, Ortmann and Schiebler, 1950). That an actual transport takes place by a proximo-distal current of axoplasm was suggested by Drager (1950). He replaced the pituitary gland by fibrin foam in the tropical indigo snake (*Spilotes corais*) and found two to three weeks after the operation in the non-living implant granules of a stainable material similar to that elaborated by the neurosecretory cells. Histological observations in the garter snake indicate that the mechanism is the same as in the tropical indigo snake. The pituitary stalk of the garter snake contains large amounts of the stainable material (Figs. 2, 3) which is also found in the neurosecretory cells of the supraoptic and paraventricular nuclei. This material eventually accumulates in the pars nervosa of the hypophysis.

A small number of fibers which arise from cells in the anterior portion of the nucleus paraventricularis and run anteriorly and dorsally toward the commissura pallii posterior (Kappers, Huber and Crosby, 1936, p. 1347) and the paraphysis (Figs. 4, 5), constitute another, hitherto unknown, connection along which neurosecretory granules may be traced. The stainable material accumulates in the commissura pallii posterior in much the same way as in the pituitary stalk (Figs. 6, 7); the amounts of the material vary from individual to individual (Figs. 5, 6). Fibers from the commissura pallii posterior carrying small blue-staining granules can be traced into the paraphysis. No accumulation of the stainable substance seems to take place in the paraphysis; here the granules appear to fade out.

Although the quantities of stainable material involved in the pathway from the paraventricular nucleus to the paraphysis are small by comparison with those seen in the hypothalamo-hypophyseal system, a similar principle seems to obtain in both cases. This similarity of the dorsal and ventral outlets for the secretory products of



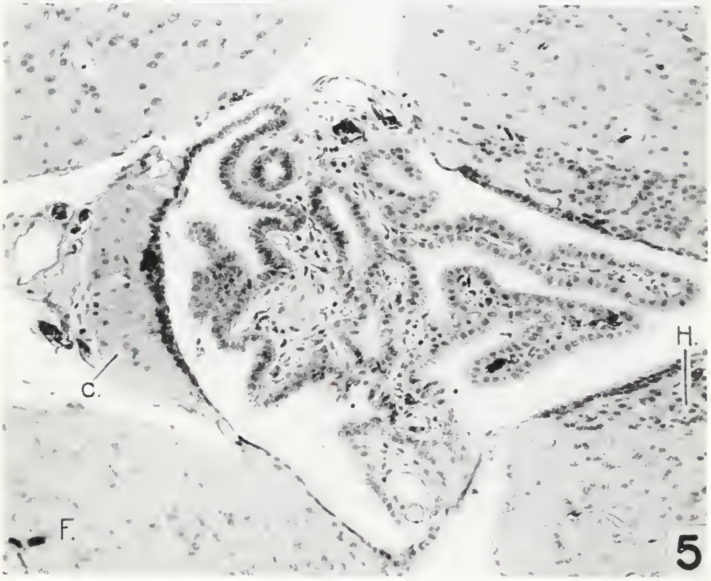
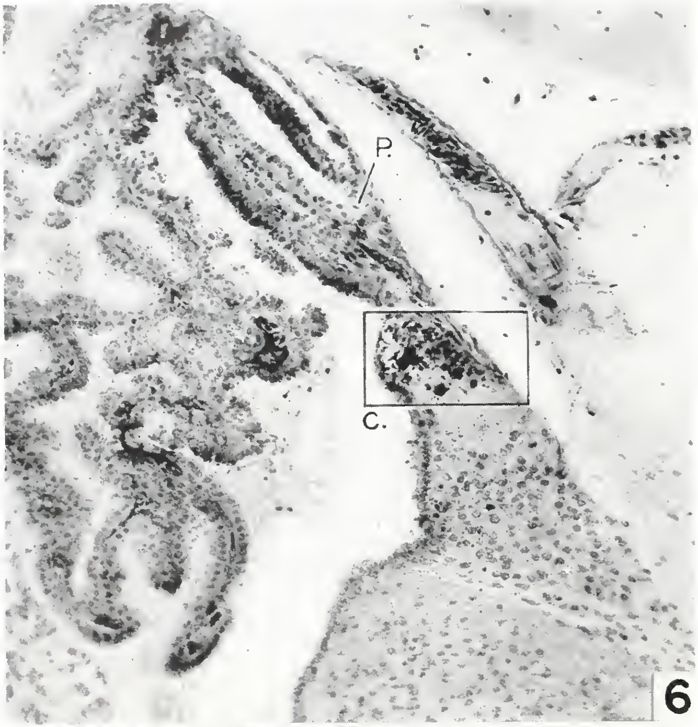


FIGURE 4. Sagittal section through diencephalon. × 40. C., Commissura pallii posterior; E., Epiphysis; M., Midbrain; O., Optic chiasm; P., Paraphysis; PN., Paraventricular nucleus; S., Stalk of pituitary.

FIGURE 5. Basiparallel section through the epithalamus. Note the small amount of stainable material in the commissura pallii posterior as compared with the large amount in the animal shown in Figures 6 and 7. × 180. C., Commissura pallii posterior; F., Forebrain; H., Habenular ganglion.



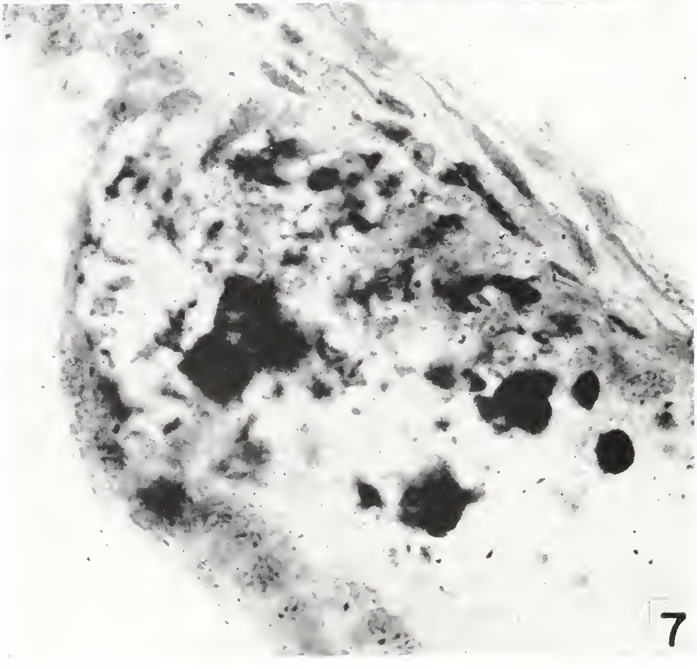


FIGURE 6. Sagittal section through the epithalamus. × 180. C., Commissura pallii posterior; P., Paraphysis.

FIGURE 7. Accumulation of stainable material in the Commissura pallii posterior in the same animal shown in Figures 4 and 6. × 1000.

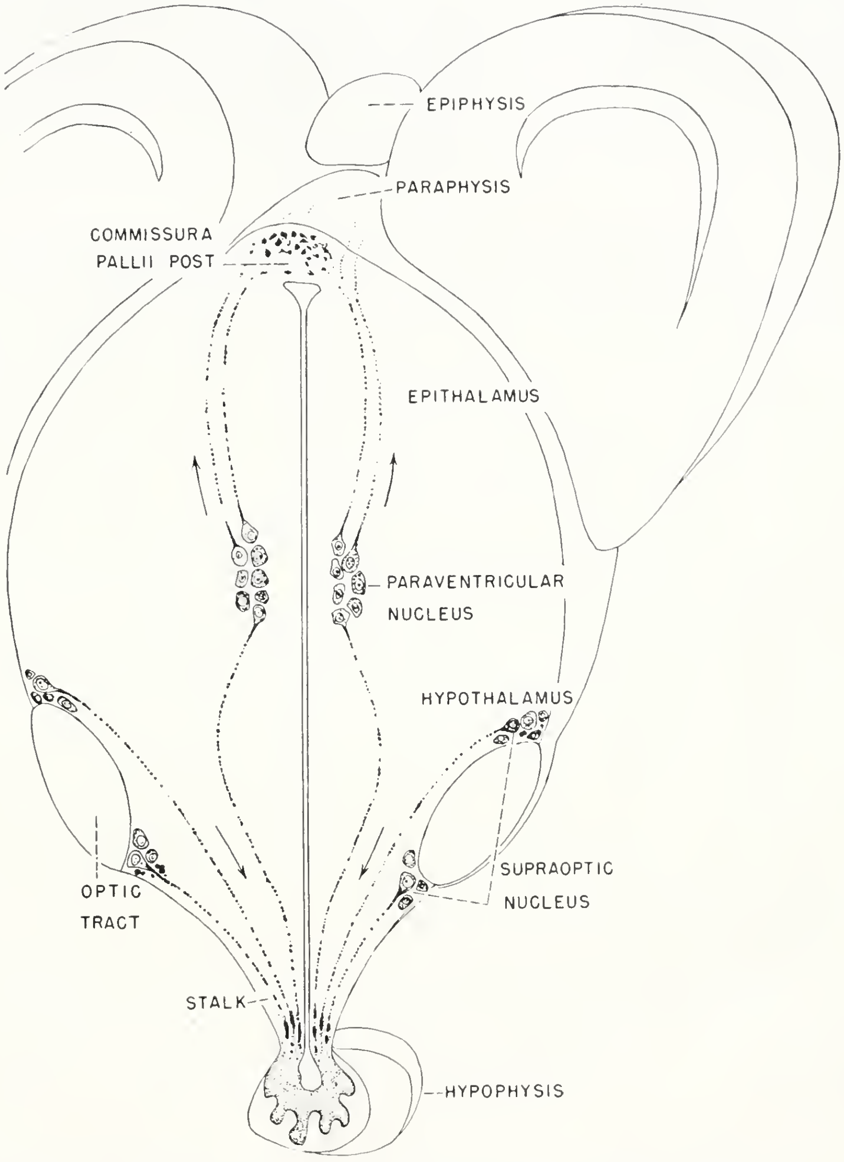

EPIPHYSIS
PARAPHYSIS
COMMISSURA PALLII POST
EPITHALAMUS
PARAVENTRICULAR NUCLEUS
HYPOTHALAMUS
SUPRAOPTIC NUCLEUS
OPTIC TRACT
STALK
HYPOPHYSIS

FIGURE 8. Diagram of cross section through the diencephalon of Thamnophis sp. The irregularly spaced dots represent granules of stainable material which mark the axons of neurosecretory cells of the paraventricular and supraoptic nuclei. These granules pass from the neurosecretory cells by way of the axons to (a) the neurohypophysis and (b) the commissura pallii posterior and the paraphysis. The arrows indicate the direction of flow.

the hypothalamus is diagrammatically illustrated in Figure 8. In both cases fine granules of the stainable material "migrate" along the processes of the neurosecretory cells, the larger part going in the direction of the pituitary, a smaller part in the direction of the paraphysis. In the pituitary stalk and in the commissura pallii posterior, the granules coalesce to form large masses of stainable material. In the pars nervosa of the pituitary and in the paraphysis, again we find delicate nerve fibers with the characteristic beadlike arrangement of small blue-staining granules.

Discussion

Tilney (1938) enumerates 10 cerebral "glands," namely (1) the paraphysis, (2) the periphysis, (3) the epiphysis, (4) the mesophysis, (5) the metaphysis, (6) the lateral chorioideal glands, (7) the mesial chorioideal gland, (8) the caudal chorioideal gland, (9) the hypophysis, and (10) the saccus vasculosus. Tilney's outline is useful in that it calls attention to the fact that the pituitary is not the only, but one of several glands which are connected with the brain. His summary may serve as a starting point for the exploration of this interesting and rather obscure field, but after some study it appears that the list of "glands" was drawn up rather schematically and requires revision in several respects. For instance, a large structure overlying the medulla oblongata in the ganoid fishes, which Tilney calls the metaphysis, is not a gland at all, but myeloid tissue (Scharrer, 1944). Similarly, the inclusion of the saccus vasculosus of the teleost fishes among the "glands of the brain" seems questionable. The occurrence of the saccus vasculosus in deep sea fishes, together with its finer structure, innervation, and vascularization suggest that this organ may be concerned with the perception of pressure differences and the maintenance of intracranial pressure equilibrium in vertical movements (Dammermann, 1910; Scharrer, 1948).

Even the criteria which are used in classifying the neurohypophysis as a "gland of the brain" are now under scrutiny. As was pointed out above there is evidence that the stainable material and the extractable hormones of the neurohypophysis are produced by secreting nerve cells in the diencephalon. According to this view the pars nervosa of the pituitary gland is a site of storage rather than of production of hormones and of microscopically visible material.

A relationship between the hypothalamus and the paraphysis is here suggested which so far has been observed only in the genus of snakes used in this study, but which deserves further exploration in other groups of animals. In the garter snake this relationship looks like a mirror image of that which obtains between the hypothalamus and the hypophysis (Fig. 8). It is at present not clear whether the substances produced by the neurosecretory centers of the diencephalon are stored in their active form in the pars nervosa of the pituitary and the commissura pallii posterior, or whether they represent only antecedents necessary for the synthesis of hormones. It also remains undecided at present whether these structures, the pars nervosa and the commissura pallii posterior, together with the paraphysis, serve no other purpose than to store and release the substances which they receive from the neurosecretory cells or whether they themselves produce additional material.

Summary

In snakes, as in other vertebrates, the neurosecretory cells of the supraoptic and paraventricular nuclei discharge their products by way of their axons into the pars

nervosa of the pituitary gland. In the garter snake (Thamnophis sp.) another, hitherto undescribed neurosecretory pathway exists: secretory granules can be traced along processes of cells of the paraventricular nuclei to the commissura pallii posterior and the paraphysis. The granules accumulate in the commissura pallii posterior in a manner similar to that observed in the stalk of the pituitary.

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CHARACTERISTICS OF THE RETINAL ELECTRIC RESPONSE OF THE OCELLI OF LIMULUS

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The median ocelli of *Limulus polyphemus*, located one on each side of a spine on the dorsal and anterior surface of the carapace, are well suited for investigations into the electro-physiological properties of photoreceptors. These small clusters of sense cells, about 50–80 in number, can be dissected free of the cornea and surrounding connective tissue quite readily. The free ocellus, about 0.5 mm. in diameter, can then be drawn into a tapering capillary tube. Such a preparation, providing it is not subjected to unreasonable abuse, will give reproducible electrical responses upon illumination for periods up to six hours.

The data presented below were obtained from ocelli treated in the manner just described. A capillary containing the ocellus was sealed into a small hole in the partition which separated two compartments of a plastic electrode chamber. The light-receiving end of the ocellus was in contact with sea water of the forward compartment and the terminal portion of the nerve was in contact with sea water in the rear compartment. Each compartment contained a silver-silver chloride electrode in contact with the sea water.

The light source used in this experiment was a 500 watt tungsten filament projection bulb. At the focal point the maximal intensity was 27,000 foot candles and this was considered unit intensity. To cool the light beam it was passed through 8 centimeters of water and a heat-absorbing filter. The intensity of the light beam was reduced by Wratten neutral tint filters. The duration of the light flash was controlled by a photographic shutter. A portion of the light beam was diverted to a photocell, the output of which was recorded as the stimulus signal. The nominal exposures of the shutter were calibrated by photographing a cathode ray beam deflected by the output of the photocell.

The potentials appearing across the eye on illumination were fed into high gain, condenser-coupled amplifiers and recorded on an ink writer. The recording paper speed was 25 centimeters per second; the paper speed was calibrated with a 60 cps. signal. The ink writer responded faithfully to calibrating signals up to 60 cycles per second. The deflections of the writing pens were calibrated with square wave voltage pulses and a 60 cycle per second signal after each exposure. The time constant of the amplifiers was 0.5 second. It is felt that the distortion of the wave form of the retinal action potentials caused by the 0.5 second time constant does not seriously affect the results obtained nor the interpretation of the results.

After dark-adaptation was complete, a flash of light of the lowest intensity to be used was admitted and the response recorded. For relatively low intensities and/or short durations (less than 0.1 second) of illumination, 10 minutes between flashes were found to be sufficient for recovery. For higher intensities and/or for longer durations (more than 0.1 second) of illumination, it was necessary to allow 15 to 20 minutes between flashes.

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RESULTS

The wave form of the response

The time course of the retinal action potential obtained upon brief illumination of an excised median ocellus is indicated in Figure 1. The smooth contour of the response is similar to the second response curve obtained from the lateral eye of Limulus (Graham, 1932; Wulff, 1950). Light flashes of very low intensity (0.027 f.c. or less) often elicit responses of irregular contour (Fig. 1, right). As the flash duration is increased, the response spreads out in time and the irregularity increases. If the intensity of illumination is increased, the irregularity of the response disappears.

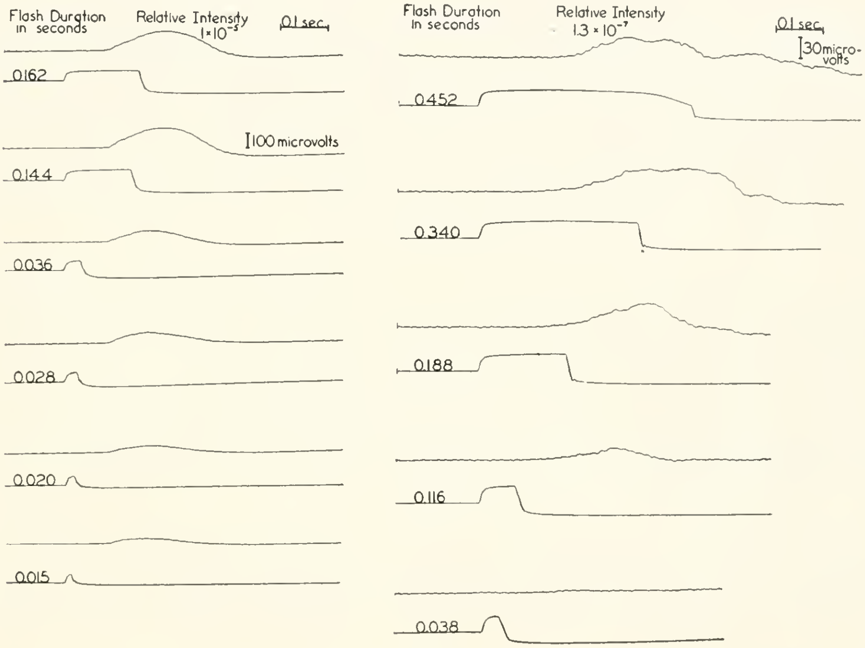

FIGURE 1. Right: Photographs of retinal action potentials elicited from an isolated ocellus illuminated with low intensity for varying durations. The duration in seconds is given on the lower tracing of each pair of records, which is the record of the output of the photocell. The ocellus was maximally dark adapted before each flash.

Left: Photographs of retinal action potentials from an ocellus illuminated with a moderate intensity for varying durations. Records marked as above. Refer to text for further detail.

The effect of light flashes of constant duration and variable intensity

A light stimulus of the lowest useful intensity generally elicits a response with crest magnitudes of about 10 microvolts. As the intensity of illumination is increased, the response magnitude increases also, the relation being such that the

Table I

Latent periods and crest times obtained during a typical experiment with an excised median ocellus of Limulus. Relative intensities are at the top of each column. All times are in milliseconds. The means and their standard deviation are given underneath particular columns

Flash Duration msec.	1×10^{-6}		1×10^{-5}		1×10^{-4}		1×10^{-3}		1×10^{-2}		1×10^{-1}		1×10^{0}	
	Latent Period	Crest Time	Latent Period	Crest Time	Latent Period	Crest Time	Latent Period	Crest Time	Latent Period	Crest Time	Latent Period	Crest Time	Latent Period	Crest Time
17	128	96	80	80	72	92	52	84	40	72	28	36	23	32
21	120	60	85	92	73	83	56	76	40	64	29	35	23	32
32	118	94	89	112	68	84	56	84	—	—	28	32	23	32
42	104	100	88	124	72	84	56	88	40	68	—	—	—	—
101	108	125	91	120	71	77	56	92	40	76	—	—	—	—
256	100	200	84	129	74	82	—	—	—	—	—	—	—	—
Mean	113		86		72	84	55	85		70	28	34		
Standard Deviation	±9.8		±3.6		±1.9	±4.4	±1.6	±4.8		±6.3	±0.6	±1.6		

magnitude increases linearly with the logarithm of the intensity. The largest response magnitude recorded from an ocellus exposed to a flash of unit intensity (27,000 f.c. at the surface of the ocellus) was 5 millivolts.

The latent period of the electric response generally decreases as the intensity increases. The behavior of both magnitude and latent period of the response of the ocellus are in agreement with similar characteristics of the lateral compound eye of Limulus (Wulff, 1950).

The effect of light flashes of constant intensity and variable duration

When the intensity of the light stimulus is maintained constant and the duration is increased, a series of responses such as is illustrated in Figure 1, left, may be

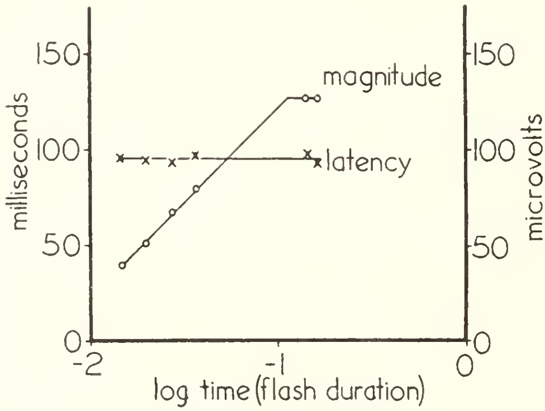

Figure 2. Magnitude of the crests and latencies of the responses of Figure 1, left, plotted as a function of the common logarithm of the flash duration. Refer to text for further detail.

obtained. The latent period of the responses is constant for all flash durations at the same intensity level (Fig. 1 and Table I). It is only when the intensity level is changed that the latent period changes.

The magnitude of the responses illustrated in Figure 1, left, gradually increases to a maximal value which is reached when the flash duration is about 0.1 second (see Fig. 2). Light flashes of duration longer than 0.1 second do not produce any further increase in magnitude. The influence of flash duration on response magnitude has been determined for the grasshopper eye by Hartline (1928).

Histological examination of ocelli

Ocelli used in these experiments were prepared for histological study by fixing in Bouin's solution, imbedding in paraffin, sectioning and staining with thionine ($C_{12}H_9N_3S$). This stain has a marked affinity for the spherical rose-colored inclusions richly distributed throughout the cytoplasm of the sense cells. In suitable preparations cut in cross section, the numbers of sense cells were counted. The results indicate that ocelli of the size used in these experiments contain between 50 and 80 sense cells.

Discussion

Although the electrical response of the ocellus of Limulus does not differ significantly from the electrical responses of other photoreceptors, certain of the observations lead to a critical evaluation of some of the characteristics of the retinal action potential.

The magnitude of the retinal action potential

The increase in the crest magnitude of the retinal action potential of the dark-adapted photoreceptor, produced by increasing the intensity of illumination or by increasing the duration of exposure at a given intensity, has been frequently reported (Kohlrausch, 1931; Hartline, 1928; Granit, 1947; Wulff, 1950). The growth of response magnitude seems to be characteristic of photoreceptors, yet no adequate explanation has been advanced to account for the tremendous range of responses of which photoreceptors are capable. The observations reported here suggest that the increase in magnitude of the electrical response of the median ocellus of Limulus may be primarily the result of graded responses on the part of the sense cells, with the added possibility that sense cell recruitment may occur at low intensities of illumination.

That the sense cells of the median ocellus of Limulus can produce responses of graded magnitude is indicated by the following observations. The maximal response recorded from an isolated ocellus was of the order of 5 millivolts. Minimal response magnitudes that could be measured with reliability were of the order of 0.01 millivolt. Should 0.01 mv. represent the maximal (all or none) response of a single sense cell then, assuming the best possible conditions for summation, the number of sense cells required to produce a response of 5 mv. would be 500. Actual counts of sense cells in ocelli used range between 50 and 80, a number entirely inadequate to account for the observed results unless each sense cell is capable of producing a response whose magnitude varies with the intensity, the duration of illumination or both.

The recruitment of sense cells by light flashes of long duration incident on an excised median ocellus of Limulus is illustrated in Figure 1, right. The extreme temporal dispersion of discrete electrical waves indicated in Figure 1 is observable only at low levels of illumination. Increasing the intensity of illumination generally produces a smooth response regardless of the exposure (Fig. 1, left). However, the crests of the smooth responses shift in time as the light flashes increase in duration so that the crest time, measured from onset of response to the crest, increases (Table I). The shift in crest time is apparent only at the lower intensities of illumination (Table I) and it is assumed that recruitment of sense cells occurs at those intensities where the crest time increases with increasing flash duration.

The retinal action potential and the optic nerve discharge

It has been suggested (Hartline, 1935; Wulff, 1943; Granit, 1947) that the retinal action potential generates local currents which initiate impulses in the optic nerve. In the light of such an hypothesis a large retinal action potential would set up more intense local currents than a smaller potential and hence the resultant nerve impulse discharge might begin sooner in time and be more intense, *i.e.,* the frequency of the impulses might be greater. Hartline (1934) has measured the characteristics of the nerve impulses discharged into a single optic nerve fiber in response to light flashes of different intensities and durations. The results of Hartline's observations that pertain to this discussion are: 1) The frequency of the first three impulses of the train of impulses increases with increasing flash duration, reaching a value that is roughly constant at flash durations of 0.1 second and higher; 2) The latency of the optic nerve discharge decreases as the flash duration increases, reaching values roughly constant for flash durations of 0.1 second and higher. Assuming for the moment that the characteristics of the ocellus of Limulus are applicable to the lateral eye, then the results of Hartline's experiment are in accord with the hypothesis that the retinal action potential initiates activity in the optic nerve because: 1) the direction of the changes in latency and frequency of the optic nerve discharge produced by light flashes of increasing duration is in harmony with the increase in magnitude of the retinal action potential; and 2) the salient characteristics of both the retinal action potential and the optic nerve discharge are constant for light flashes of durations of 0.1 second and higher. Very recently it was demonstrated that the response characteristics of the lateral eye of Limulus are in accord with the characteristics of the ocellus.

The constant latent period

The results reported above indicate that (within the limits of exposures available) the latent period of the retinal action potential is an inverse function of the intensity of illumination and is quite independent of the duration of illumination. Since the magnitude of the response increases to a maximal value at any given intensity for flash duration of 0.1 second or greater and the latent periods of the responses are quite constant (Table I), the possibility is suggested that the onset of the response and the magnitude of response may be controlled by separate events in the sense cells. The validity of this suggestion must be tested by further investigation.

Another interpretation of these results may be based on the existence of a sensitization period, first described by Hecht (1918, 1919). According to Hecht, the sensitization period is that period during which the photoreceptor must be illuminated in order to produce a response. The duration of such a sensitization period in the Limulus ocellus might well be shorter than the shortest flash duration available, and it is conceivable that this sensitization period may determine the latency, *i.e.,* at any given intensity, a response of fixed latency either will occur or else there will be no response at all. Data obtained from the ocellus of Limulus at low levels of illumination (Fig. 1) indicate that the response magnitude for short exposures tends to approach the abscissa (zero microvolts) very gradually; there is no suggestion of a sudden drop that might be expected if a sensitization period actually exists.

SUMMARY

1. The electrical responses to illumination were obtained from isolated median ocelli of *Limulus polyphemus* mounted in a capillary tube filled with sea water. These preparations, maintained in a state of dark adaptation, were periodically illuminated at different intensities for different durations.

2. For the most part, the reported data are in accord with similar data obtained from the lateral compound eye of Limulus and the compound eyes of grasshoppers. It was observed that the latent period of the responses elicited by light flashes of different durations at any one intensity is constant; the only factor seemingly affecting the latent period is the intensity.

3. The increase in electrical response magnitude produced either by increasing the intensity and/or the duration of illumination, which is typical of many photoreceptors, is accounted for on the basis of graded responses on the part of sense cells. The possibility exists that recruitment of sense cells is a contributing factor at low intensities of illumination.

4. Comparison of data obtained from the median ocellus of Limulus with data obtained from single optic nerve fibers of the lateral eye of Limulus indicates a parallelism which is in accord with the hypothesis that the retinal action potential sets up local action currents which initiate impulses in the optic nerve.

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OXIDATION-REDUCTION INDICATOR PATTERNS IN REGENERATION OF THE FORELEG AND TAIL OF TRITURUS TOROSUS LARVAE

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In various hydroids, *e.g.*, *Corymorpha* and *Hydra*, reconstitution after removal of a part is largely or entirely by redifferentiation of tissues not directly involved in the injury and often at a considerable distance from it. In the planarian some new tissue which may be regarded as a blastema develops at the surface of injury and gives rise to head and posterior end but more or less extensive redifferentiation of other parts at various distances from the region of injury also occurs. In many cases almost the entire isolated piece undergoes reconstitution into some other part. In these organisms a marked increase in rate of oxidation and reduction of indicators appears at the injured surface almost immediately following removal of a part, *e.g.*, by section, and extends a variable short distance into the intact tissues. This is evidently an injury reaction. As reconstitution progresses, the region of redifferentiation also shows increased reactivity to the indicators, in many forms even before morphological differentiation is evident. *Corymorpha* is a particularly good example of such change in physiological condition preceding morphogenesis (Child and Watanabe, 1935; Watanabe, 1935). In the planarian there is also alteration of indicator pattern adjoining the surface of injury as an injury reaction and in other parts during the following reconstitution but the new tissue regenerating from the cut surface shows more activation than the redifferentiating parts (Child, 1948a).

The reconstitutional capacity of legs, tail, gill filaments and balancers in urodele amphibians has long been known. These and many other cases of reconstitution are commonly called regeneration because regeneration appears to be accomplished by development of a blastema on the surface of injury, apparently involving more or less dedifferentiation of cells with proliferation, growth and redifferentiation, usually into the part removed, and apparently with little or no alteration or redifferentiation of other parts. It has seemed of some interest to attempt to determine whether or to what extent alteration of oxidation-reduction indicator pattern is involved in a case of this sort.

MATERIAL AND METHODS

For leg regeneration *Triturus* larvae 12–20 mm. in length were used, in most cases larvae in which one or more toes were developing on the foreleg. Legs were amputated at various levels. For tail regeneration larvae ranging from 10 to 16–18 mm. were used with different levels of amputation. The experimental larvae were fed every 2–3 days on pieces of enchytraeids. In stocks not sufficiently fed and with more than a single individual in a container legs were frequently bitten off, usually near the foot. These provided incidental material for stages of regeneration, though time of amputation was not known. These larval stages were used because the regeneration was rapid and because pigment development was not sufficient to obscure indicator patterns.

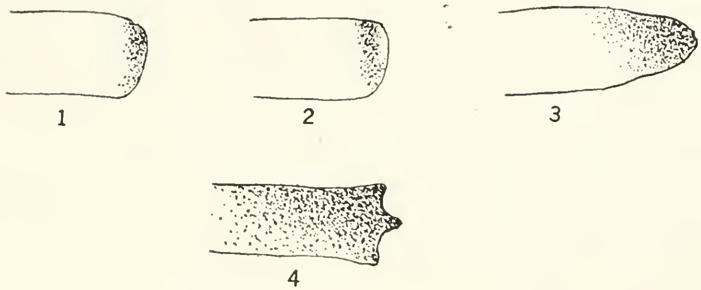
Intracellular formation and reduction of indophenol and intracellular oxidation of the dyes Janus green (diazine green) and methylene blue in solutions reduced by sodium hydrosulphite were used for intracellular oxidations. Following intracellular oxidation (primary oxidation) intracellular reduction of indophenol was obtained by addition of sodium hydrosulphite and of the dyes by addition of more hydrosulphite after primary oxidation. The indophenol reaction was used with very much lower concentrations of the reagents, para-aminodimethyl aniline (dimethyl paraphenylene diamine) and α-naphthol than in the original Nadi reaction. Use of the extremely low concentrations of the indophenol reagents has been described at length in other papers (*e.g.,* Child, 1948a; 1951). The intracellular reaction occurs in a very wide range of low concentrations of the reagents and with the very low concentrations it becomes an exceedingly sensitive indicator of certain differentials in physiological conditions and their changes in living material without appreciable injury but the most satisfactory concentrations for any particular material have been determined by trial. In the present cases, rather rapid reaction being desired, aniline 1/8000–1/16000, assuming that the sample is pure, and naphthol in much lower unknown concentration were used. Enough of the naphthol dissolves in water without heating and without addition of NaOH to give the reaction readily but amount of naphthol dissolving and actual concentrations used are unknown. Differential reduction of intracellular indophenol by sodium hydrosulphite and re-oxidation by oxygen was possible in living material. As a reducing agent sodium hydrosulphite is far superior to the other highly toxic reducing agents used in earlier studies of indicator pattern. Only very small amounts are required for reduction of indophenol or dyes; a small fraction of a milligram in one ml. of water will reduce intracellular indophenol or oxidized dyes in solution or within cells. Hydrosulphite is non-toxic in concentrations much higher than those required for reduction, except that long exposure without re-oxidation may inhibit or kill by lack of oxygen. For intracellular oxidation in reduced dye solution excess of hydrosulphite beyond that required to reduce the dye should be avoided. Excess of the reducing agent may prevent intracellular oxidation. Patterns of intracellular indophenol reaction and of dye oxidation in reduced solution and patterns of intracellular indophenol and dye reduction do not differ appreciably, *i.e.*, regions which oxidize more rapidly also reduce more rapidly than others until high intracellular concentrations of indicators retard or completely prevent reduction differentially.

Figures are necessarily diagrammatic attempts to indicate the differentials in indicator patterns following section and during the earlier stages of regeneration of foreleg and tail.

The kindness of Dr. V. C. Twitty in permitting use of material from his stocks is gratefully acknowledged.

FORELEG REGENERATION

Almost at once after amputation, at least as soon as the indophenol reaction or primary intracellular oxidation of dyes became visible, color appeared at the tip of the leg stump earlier than in any other part of the body and gradually extended proximally in the stump for a short distance, usually less than a millimeter, in a decreasing gradient. This injury reaction involved not only the surface of the stump but also internal tissues including the bone which sometimes protruded from the stump. Also the anteroposterior and dorsiventral gradient pattern of the leg (Child, 1943b) usually became distinguishable in slightly more rapid color reaction anteriorly and dorsally in the stump. The injury pattern with anteroposterior differential is indicated in Figure 1. The dorsiventral differential is similar. Patterns of indophenol reaction and primary intracellular oxidation of dyes are the same. Later more or less reduction of indophenol and dyes often occurred at the tip of the stump without external oxygen decrease and with the same anteroposterior and dorsiventral differential as the oxidase reaction (Fig. 2). It was noted earlier (Child, 1943b) that such secondary differential reduction often occurred, at least distally, in intact developing legs, gill filaments and balancers without external oxygen decrease and in oxidized dye solutions. Its occurrence suggests high oxidase activity. During the first 2–3 days following amputation the injury reaction appeared to decrease slightly in some cases. An injury reaction also occurred adjoining the cut surface of the distal amputated part of the leg with the same anteroposterior and dorsiventral pattern.



FIGURES 1–4. Regeneration of the larval foreleg. Figures 1 and 2, injury reaction, as indicated by intracellular indophenol: Figure 1, early injury reaction, the anteroposterior pattern of the leg appears in the greater reactivity of the anterior side, uppermost in the figure; Figure 2, 1½–2 hours after amputation, intracellular reduction of indophenol at the extreme tip of the stump, without external oxygen decrease. Figure 3, twelve days after amputation just above knee, primary intracellular oxidation of Janus green to red diethyl safranin in solution reduced to colorless by sodium hydrosulphite, longitudinal and anteroposterior gradient patterns of the leg are evident in the regenerate and increased reactivity extends proximal to the level of amputation. Figure 4, sixteen days after amputation just below knee, indophenol reaction, three toes develop almost at the same time, the longitudinal gradient decreases proximally from the tips of the toes, the anteroposterior pattern from the anterior (upper) side of the figure.

During the earlier stages of regeneration the indophenol reaction and primary dye oxidation in reduced solution were more rapid in the regenerating tissue than in more proximal levels of the leg and other regions of the animal. Anteroposterior and dorsiventral gradients were evident in the regenerating tissue, as well as in the more proximal intact parts and the increase in reactivity appeared to extend a short distance into the intact part of the leg proximal to the level of amputation (Fig. 3). The reduction differentials following the primary oxidative reaction were similar until intracellular concentrations of indophenol or oxidized dyes became so high that reduction was differentially retarded or completely inhibited in the most susceptible regions in which the primary oxidative reaction was most rapid. Under these conditions more or less reversal of the color gradient pattern occurred.

Sixteen days after amputation approximately at the knee early stages of 3 toes were visible in some individuals and the exact boundary between the regenerated tissue and more proximal regions was becoming less distinct. The increased reactivity of the regenerating region was still evident and still appeared to extend somewhat proximal to the level of amputation. In the regenerated region the reactivity decreased proximally from the tips of the toes and continued without sharp boundary into the pattern of the uninjured region (Fig. 4). In this appearance of three toes at almost the same time the regenerating leg differs somewhat from the order of toe development in the uninjured leg. In those legs one toe appears in the dorsal or anterodorsal region, *i.e.*, from the highest levels of the gradient pattern of the leg, a second toe, slightly later from a more ventral level and a third, still later, also dorsal but becoming posterior to the first. In the regenerating leg the two toes developing from the higher gradient levels differ very little in rate or stage of early development and in reactivity. The third toe, uppermost in Figure 4, shows slightly less reactivity. In later stages the middle toe of Figure 4 elongates more rapidly than the others. At the stage of Figure 4 early development of the foot is also becoming evident in the slightly broader distal region of the regenerate. The anteroposterior and dorsiventral differentials in the pattern of the leg become evident progressively from the tips of the toes proximally through the regenerate into the proximal region of the leg. Progress of reduction is in the same direction until toxic effect retards or inhibits reduction distally, first of all in the toes. With rapid oxygen increase in the solution re-oxidation pattern is like the pattern of primary oxidation but with slight or slow oxygen increase is reversed. The superficial tissues of the leg were injured by the indicators earlier than the internal tissues, so that it was possible at a certain stage of toxic effect to observe more or less reversal of the original reduction gradient pattern superficially with persistence of the original pattern in the internal tissues. In later stages of leg regeneration the pattern gradually approaches that of the intact developing leg, as described elsewhere (Child, 1943b), consequently further consideration of the regeneration pattern is regarded as unnecessary.

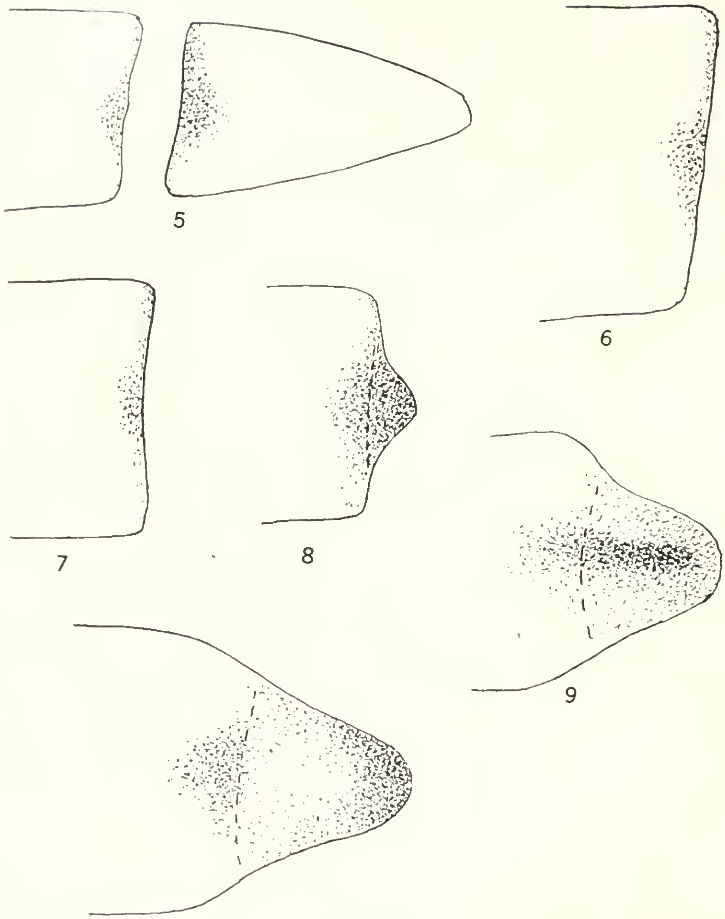
Regeneration of the Tail

In an earlier study of indicator patterns in early amphibian development (Child, 1948b, pp. 95–99) it was noted that when larval stages earlier than those used in the present paper were completely separated into successive pieces by transverse section at different levels it was found that the reactivity, as indicated by intracellular indophenol, intracellular oxidation of dyes in reduced solution, and intracellular reduction of both indophenol and dyes by hydrosulphite adjoining both anterior and posterior cut surfaces, decreased from anterior to posterior levels of section of the body and tail and from dorsal to ventral at each level of section. In each piece the reaction was more rapid and the spread of the reaction from the cut surface was greater in the posterior direction from the anterior cut surface than in the anterior direction from the posterior surface and reactivity and spread were greater at the anterior end of each piece than at the posterior end of the piece immediately anterior to it, *i.e.,* at virtually the same body level reactivity and spread were less when the cells concerned were at the posterior end of a piece than when they were at the anterior end of the following piece. In the somewhat later larval stages used as material for tail regeneration transverse sections at various levels of the tail and at more anterior levels show the same differences in the injury reactions with differences in level of body or tail and in anterior or posterior position in the piece as the earlier stages. A single example following section of the tail halfway between the anus and the tip is sufficient to illustrate the point (Fig. 5). This is actually a case of intracellular reduction of oxidized Janus green to the red diethyl safranin, the parts reducing most rapidly being shaded for comparison with figures showing oxidase reactivity. The figure shows the condition 15 minutes after section: reduction to red is more rapid and the spread posteriorly of reduction in the posterior separated piece of the tail is greater than at the posterior end of the tail stump, both in the region of the nervous system and muscles, the "axial" region of the tail and in the dorsal and ventral fins, much less in the fins than in the axial region but slightly greater in the dorsal than in the ventral fin in both the stump and the separated tip. In earlier stages of the reaction a slight gradient, decreasing ventrally, is usually distinguishable in the fins.

Following transverse section at a level approximately one mm. posterior to the tip of the early hindleg the injury reaction is more rapid and extends farther anteriorly in both the body region of the stump and the fins (Fig. 6) than after section at more posterior levels (Fig. 7, also the reaction in the stump of Fig. 5). In short, the relations of these injury reactions to the gradient pattern of the individual are the same in these later larval stages as those previously observed in earlier stages after section at various levels. The injury reaction following section in animals under chloretone anaesthesia appeared to be somewhat less rapid and less extensive than in unanaesthetized larvae. The injury reaction in the tail stump is more rapid than in any other part of the larva.

Figure 8 is an example of indicator pattern in the regenerating tail 7 days after amputation near the middle. There are of course individual variations in rate of outgrowth of tissue other than those resulting from amputation at different levels. The outgrowth is chiefly in the middle region in which the nervous system, spinal cord and muscles develop. Dorsally and ventrally the outgrowth decreases. At this stage the outgrowth shows merely a general gradient decreasing anteriorly without visible distinction of particular parts. It is noted as a point of some interest that the increased reactivity extends into tissues anterior to the level of section with a further decrease anteriorly of the gradient for a distance almost equal to the length of the regenerate; even in this region the reactivity is greater than in other parts of the larva. In general, rate of tail regeneration and to some degree the increase in reactivity in the regenerate and anterior to the level of section decrease as level of section becomes more posterior. Detailed description of such differences seems unnecessary.

In more advanced stages of regeneration individual differences in rates of outgrowth and in conditions in the tissues of the outgrowth appear. For example, in Figure 9, 17 days after amputation, the usual gradient, decreasing anteriorly, appears but the developing axial structures in the regenerate show much greater reactivity than the rest of the outgrowth, also with a gradient decreasing anteriorly.



FIGURES 5–10. Regeneration of the larval tail. Figure 5, injury reaction following transverse section at middle of tail, as indicated by intracellular reduction of Janus green, 1/30,000, to red diethyl safranin after staining by the oxidized dye; rate of reduction is higher than in any other part of the animal and its extent, both in the caudal axial region and in the fins, is greater posterior than anterior to the level of section. The indophenol reaction pattern is the same.

FIGURES 6 and 7 show the difference in rate and extent anteriorly of the injury reaction in caudal axial region and fins, as indicated by indophenol, after section at an anterior tail level (Fig. 6) and a level posterior to the middle of the tail (Fig. 7): reactivity is much greater and extends farther anteriorly in the caudal axial tissues than in the fins.

FIGURE 8. The indophenol reaction gradient 7 days after sections, with slight decrease anteriorly in the regenerate and further decrease in the caudal axial region anterior to the levels of section and to a lesser degree in the fins; reactivity is still greater than elsewhere in the animal.

FIGURE 9. Indophenol reaction 17 days after section; much more rapid reaction in the regenerating caudal axial region with slight decrease anteriorly, than in the regenerating fins dorsal and ventral to it: anterior to the level of section reactivity decreases anteriorly but extends farther than in earlier stages: reactivity in relation to regeneration is still greater than in any other part of the animal.

FIGURE 10. Indophenol reaction in a slightly more rapid regeneration than Figure 9, 16 days after section: after ½ hour the regenerating caudal axial region has undergone secondary

The high reactivity of this region is evidently associated with certain developmental stages of these structures. Dorsal and ventral to this region there is much less rapid reaction in the developing fins. In the axial part of the tail the increase in reactivity extends farther anterior to the level of section than in Figure 8, with decrease in the anterior direction. In another case (Fig. 10) with somewhat longer regenerate 16 days after amputation the axial structures in the regenerate reacted at first much like those in Figure 9 but secondarily reduced indophenol almost completely without external oxygen decrease, suggesting greater dehydrogenase activity than in the case of Figure 9. In the individual of Figure 10 the increased reactivity also extends a considerable distance into the tissues anterior to the level of section but without secondary reduction.

In still later stages of tail regeneration the difference between the regenerate and more anterior levels gradually decreases and disappears as rate of regeneration decreases. Greater reactivity with a gradient decreasing anteriorly, except when secondary reduction occurs, persists longer in the axial region of the tail than in the fins. Since nothing of particular interest as regards indicator patterns has been observed in these later stages it seems unnecessary to describe them at length.

Intracellular reduction of indophenol and dyes shows the same differentials in pattern until differential retardation or inhibition of reduction resulting from toxic effect of indicators, appearing earliest in regions originally of most rapid indicator oxidation, alters or may reverse more or less completely the gradient pattern. Reoxidation pattern is similar to the pattern of primary oxidation if oxygen increase following reduction is sufficiently rapid. With slow or slight oxygen increase it is the reverse of the primary oxidation pattern.

Discussion

The appearance of the anteroposterior and the dorsiventral differentials in the injury reaction on the foreleg stump is of interest as evidence that the change in condition of the cells concerned in this reaction depends to some degree on their condition preceding amputation. The increase in reactivity is somewhat greater in cells representing higher gradient levels in the intact leg than in those of lower gradient levels, consequently the gradient pattern is not obliterated in the injury reaction or in later regeneration. Either the differences in physiological condition associated with their original position in the pattern of the intact leg persist more or less completely during the increased reactivity and the probable greater or less dedifferentiation characteristic of the regenerating tissue, or else the pattern is induced in them by the differentials of the stump. The presence of the pattern in the injury reaction preceding actual regeneration perhaps indicates its persistence without induction. However, in the later regeneration and differentiation of the regenerated part its relation to the stump and the ingrowth of nerves doubtless plays a part.

The development of three toes almost at the same time in the regenerating leg, rather than in a definite sequence, as in development of the intact leg is probably to be regarded as a consequence of the higher level of metabolic activity in the regenerating

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reduction without external oxygen decrease after a primary reaction: a gradient of increased reactivity, decreasing anteriorly, is present anterior to the level of section chiefly in the caudal axial tissues without evidence of reduction: reactivity in relation to regeneration is still greater than in other parts.

leg than in ordinary development which determines decrease in time differences in progress of morphogenesis.

Following transverse section in the tail the great difference in rate and intensity of the injury reaction in the post-anal extension of the neural, spinal and muscular structures, the "axial" region of the tail, and the dorsal and ventral fins is of interest as evidence of a great difference in physiological condition in the two regions. The reaction in the caudal axial region is not only much more rapid but extends much farther anteriorly from the cut surface than the very slight reaction immediately adjoining the cut surface in the fins. However, even in the slight reaction of the fins the dorsiventral gradient is usually distinguishable in the slightly more rapid reaction of the dorsal fin, and not infrequently a decrease in rate of reaction ventrally is visible in both fins.

It is also of particular interest in relation to the general significance of gradient pattern that the injury reaction is not only more rapid in both body region and fins of the posterior separated piece of the tail than in the anterior stump but also spreads farther posteriorly in the posterior piece than anteriorly in the stump. These differences in injury pattern at posterior and anterior cut surfaces are similar to those observed throughout the entire length of earlier larval stages, separated into successive pieces by a series of transverse sections at different levels (Child 1948b, Figs. 16 and 17). These differences are obviously associated with the anteroposterior gradient pattern of the larval body. On the two sides of the same level of section the injury oxidation is more rapid and spreads farther posteriorly down, than anteriorly up the gradient.

An essentially similar relation to gradient pattern appears in many other organisms, either as a feature of reconstitutional morphogenesis, or of functional physiological activities in certain organ systems of developing or mature organisms. In isolated pieces of Hydra and the planarian the differentials in reactivity to oxidation-reduction indicators in relation to body-level and to distal and proximal, or anterior and posterior cut ends of the pieces are like those in the larva of *Triturus* (Child, 1948a). Moreover, as regards bipolar forms of hydroids it has been known for many years that hydranths or partial hydranths, developing at the aboral ends of bipolar pieces of *Tubularia* stem within certain limits of length, are usually smaller, develop less rapidly, and are often less complete, than those developing from the oral end. Similar relations appear in bipolar forms of *Corymorpha*. Also in bipolar planarian heads the head developing from the posterior cut end is often smaller, develops less rapidly than the anterior head, and may show all degrees of differential structural inhibition. In both the hydroid and planarian bipolar forms the hydranth or head developing with establishment of a new gradient opposed in direction to the original gradient, involves greater changes in organization than does development at the oral or anterior end and may be more or less inhibited by the preëxisting gradient. However, in both hydroid and planarian pieces so short that the longitudinal gradient pattern is almost completely absent and consequently has little or no appreciable effect on development, apical regions or heads at both ends develop at about the same rate and under almost the same conditions as regards gradient pattern and are usually alike or almost alike.[1]

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[1] As regards the bipolar forms of hydroids see, for example, Child (1907) and earlier literature cited and discussed there; Child and Watanabe (1935); Watanabe (1935); Child (1941; pp. 36–38, 313–321). For planarian bipolar forms see Rustia (1925); Child (1941; pp. 363–364).

The following examples of relations of functional physiological activities to gradient pattern are also of interest as indicating that morphogenetic and functional relations to gradient pattern are not fundamentally different. In the ctenophore plate row conduction of the impulse determining movement of the plates, following local mechanical or other stimulation at any level of the row, progresses much farther down than up the row. Usually it progresses down the gradient present in the row over the entire length of the row below the levels of stimulation but, unless the stimulation is extreme, only over a few plates up the gradient. The mammalian intestine possesses a longitudinal gradient pattern with high end at the pylorus, and, as Alvarez has demonstrated in a series of papers, conduction following a local stimulus at some level progresses much farther, often over the entire length down the gradient, and over a much shorter distance up the gradient. The vertebrate heart also gives evidence of somewhat similar relations (Child, 1941, pp. 327–330 and references cited there).

The much more rapid outgrowth in the caudal axial region than in the fins is what may be expected in view of the evidence of much more rapid metabolism in that region than in the fins. It is also of interest that the increase in reactivity in the axial region anterior to the level of section with a gradient decreasing anteriorly is characteristic, not only of the injury reaction but of regeneration (Figs. 8, 9 and 10). Evidently the change in physiological condition anterior to the surface of section is associated with the regenerative activity of the caudal axial region. In the fins there is also some increase in the spread anteriorly of slightly increased reactivity. It appears that even in regeneration of amphibian leg and tail which are commonly regarded as a regeneration from a blastema developing at the cut surface, the indicators give evidence of a change in physiological condition extending into tissues anterior to the level of amputation. This suggests the possibility that some degree of redifferentiation may be occurring there and that this case is perhaps not fundamentally different from reconstitution in various invertebrates, *e.g.*, planarians, which is only in part a real regeneration.

In earlier indicator studies on amphibian development (Child, 1943a, 1943b) it has been stated that the re-oxidation gradient, following intracellular reduction, is opposite in direction to the gradients of primary oxidation and reduction, *i.e.*, that regions which primarily oxidize and reduce the indicators most rapidly re-oxidize them after reduction least rapidly and that rates of re-oxidation increase as rates of primary oxidation and reduction decrease. In further work with indicators this has been found to be true when external oxygen increase is relatively slow and slight. However, when it is sufficiently rapid and great the re-oxidation pattern is the same as that of primary oxidation. Larval amphibian appendages frequently reduce indophenol and dyes secondarily, *i.e.*, after first oxidizing them, and without external oxygen decrease, suggesting high dehydrogenase activity. In re-oxidation apparently oxygen available, rather than enzyme activity, may determine the re-oxidation differential if oxygen increase is slow or slight because it becomes sufficient to permit intracellular re-oxidation earlier in regions of lower, than in those of higher oxygen uptake. With rapid and great external oxygen increase the differentials in enzyme activity become the factor determining the re-oxidation pattern. In short, two opposed re-oxidation patterns are possible, not only in amphibian development but in other organisms, one determined by external, the other by internal factors.

SUMMARY

1. Amputation of foreleg and tail at various levels is followed almost immediately by increased intracellular indophenol reaction and oxidation of dyes in reduced solution.

2. In the leg the injury reaction and the later regenerative outgrowth show the anteroposterior and dorsiventral patterns of the leg.

3. Development of the first three toes at almost the same time in the course of regeneration of the foot, rather than in the sequence characteristic of the development of the intact leg probably results from the increased metabolism in the regenerating, as compared with the intact leg.

4. In tail regeneration the injury reaction is more rapid and extends much farther anterior to the surface of amputation in the caudal axial region than in dorsal and ventral fins. Also the later regenerative outgrowth is much more rapid in and near the caudal axial region than in the fins. The reactivity gradient decreases anteriorly.

5. The increase in reactivity anterior to the level of tail amputation is present not only during the injury reaction but also during the following regeneration. Its persistence suggests that considerable physiological change may occur in this region, as well as in the regenerative outgrowth.

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AN ANNUAL HISTORY OF A POPULATION OF NEREIS DIVERSICOLOR O. F. MULLER

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Nereis diversicolor O. F. Müller is probably one of the most numerous mud-flat animals in Europe. Its tolerance of widely different environmental conditions, especially its resistance to varying salinity, is well known but not fully understood, and although a certain amount of laboratory work has been performed on this animal, it has been little studied in nature. As the rate of growth of these worms, length of life and related phenomena have hitherto been unknown, a study of the history of a single population is of interest.

Observations on a population of *Nereis diversicolor* were made at Chalkwell, Essex, on the north bank of the Thames estuary in England, during 1948–1949. Population densities were determined by taking five or six 0.25 m.^{2} samples, in each instance dug to a depth of about 40 cm. Monthly samples were taken within a fairly limited area of shore about 1000 m.^{2} in area at the upper limit of the mud-flat zone where there was an average exposure of 7 hours at each tide. A frame was used with sides 30 by 83.3 cm. enclosing an area of approximately 0.25 m.^{2} The length of the shorter side was chosen in relation to the breadth of the spade used to dig out the samples. Usually five samples were taken at random within the chosen area, which was small enough to eliminate any possible variations in numbers due to differences in tidal level, major substratum differences, or other factors, but large enough to preclude the possibility of any reduction in numbers due to over-sampling on successive occasions. The muddy sand was friable enough to make hand sorting relatively easy, and it was found that this method was as accurate as sieving. When worms were found in a fragmented condition, heads only were counted.

Worms were measured after narcotization with 10% magnesium sulphate on a wet glass sheet overlying squared paper. Although very contractile, measurement of length (excluding head and anal appendages) could be made with an accuracy of ± 0.5 cm. with worms of total length 10 cm. The measured length is probably rather greater than the real length owing to the action of the narcotic. The results have been arranged in size groups with extremes differing by 2.0 cm. Where worms were of such a length that they might have been referred to either of two adjacent size-groups, the lower group was consistently preferred. Samples taken for assessing the mean length were obtained by random digging.

I am indebted to Dr. G. E. Newell for many suggestions, and to Dr. C. Barker Jørgensen for reading the typescript.

POPULATION DENSITY

The monthly variation in numbers of worms per square metre (Table I) is shown graphically in Figure 1. The population density during the winter was fairly low, but approximated to the same value (about 55/m.^{2}) from January until March. This number represented the effective breeding population density, which contained less than 10 per cent of males (Dales, 1950). Although spawning took place in this population over a period of not more than about two weeks towards the end of February, the young worms resulting from successful fertilizations at this time did not reach a length sufficiently great to be recognised by the sampling method employed until the following June (Fig. 3). From March onwards there was a slight but significant fall in the number of worms per unit area, and this was due to the spawned, and later some of the unspawned individuals having died. Most of the unspawned females die during the summer, although some undoubtedly survive until the following winter, and although this is obscured by the increase in number of small worms in each sample in Figure 1, it is shown by the histograms (Fig. 3) for these months. By inspection of the data, the percentage of the total adult (1948) generation may be estimated at about 50 per cent in June, 22 per cent in July, and only 5 per cent in August. Unfortunately, a more accurate separation of these two components (the adult and juvenile parts of the population) was found to be impossible by the graphical arithmetic probability method. From June until August the density of the popu-

TABLE I

Monthly variation of population density

Month	Number of worms in each 0.25 m.^{2} sample							Mean no./0.25 m.^{2}	Mean no./m.^{2}
1948									
October (29:10:48)	37	32						34.5	130.0
November (25:11:48)	25	38	39	16	19			27.4	109.6
December (22:12:48)	19	30	16	25				22.5	90.0
1949									
January (23:1:49)	14	8	12	13	18			13.0	52.0
February (24:2:49)	17	8	23	13	12			14.6	58.4
March (23:3:49)	11	17	20	10	11			13.8	55.2
April (21:4:49)	4	4	7	20	23	15	7	11.4	45.6
May (27:5:49)	5	15	2	10	11			8.8	35.2
June (22:6:49)	32	6	43	27	23			26.2	104.8
July (20:7:49)	23	66	70	56	79			58.8	235.2
August (19:8:49)	110	55	86	52	48			70.2	280.8
September (16:9:49)	48	76	74	43	68			61.8	247.2
October (24:10:49)	65	34	54	43	29			35.0	140.0

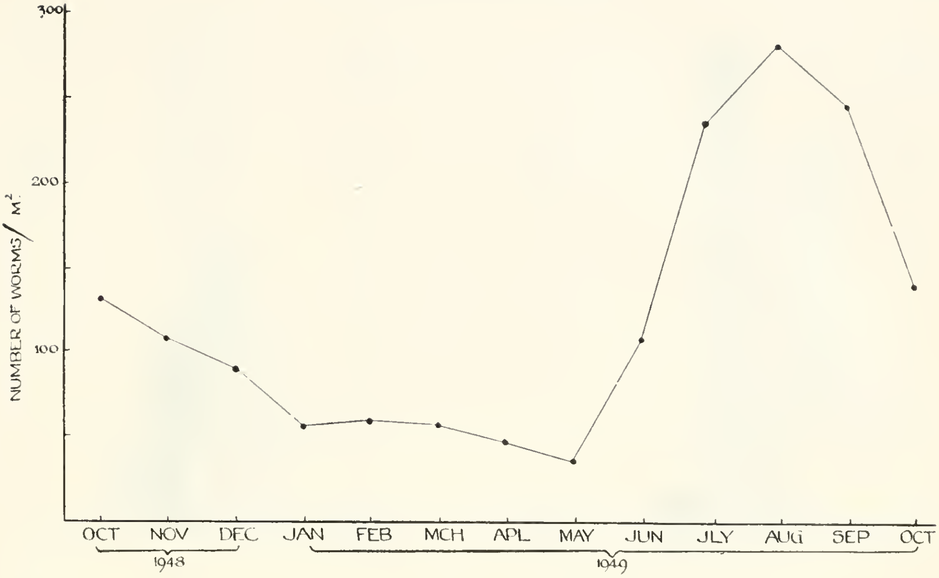

Figure 1. Monthly variation in density of population.

lation rose rapidly as the young worms became long enough to be seen in the sand. The population density reached a maximum of about 280/m.² at the end of August, after which the numbers decreased to approximately the same winter density as before. This fall in density was probably due to a thinning out of young worms, although a certain amount of emigration may also have occurred, while a small part was due to the elimination of the remnants of the previous generation.

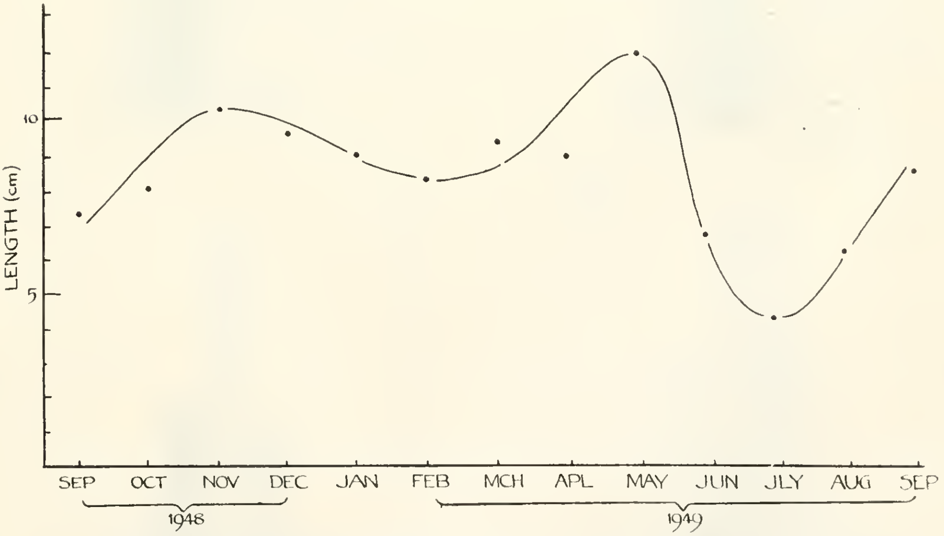

Figure 2. Monthly variation of mean length.

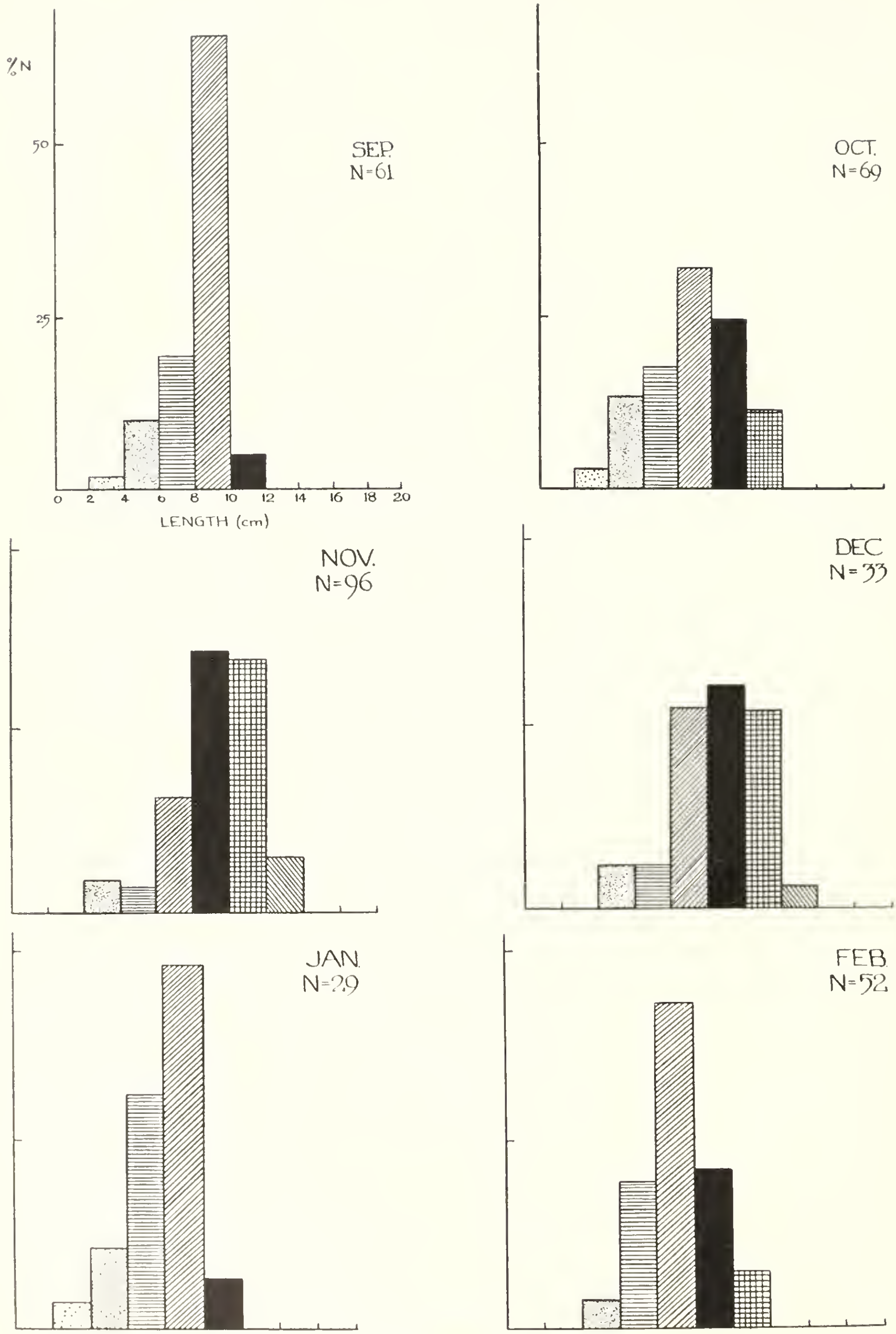

FIGURE 3. Histograms illustrating the monthly size distribution throughout the year.

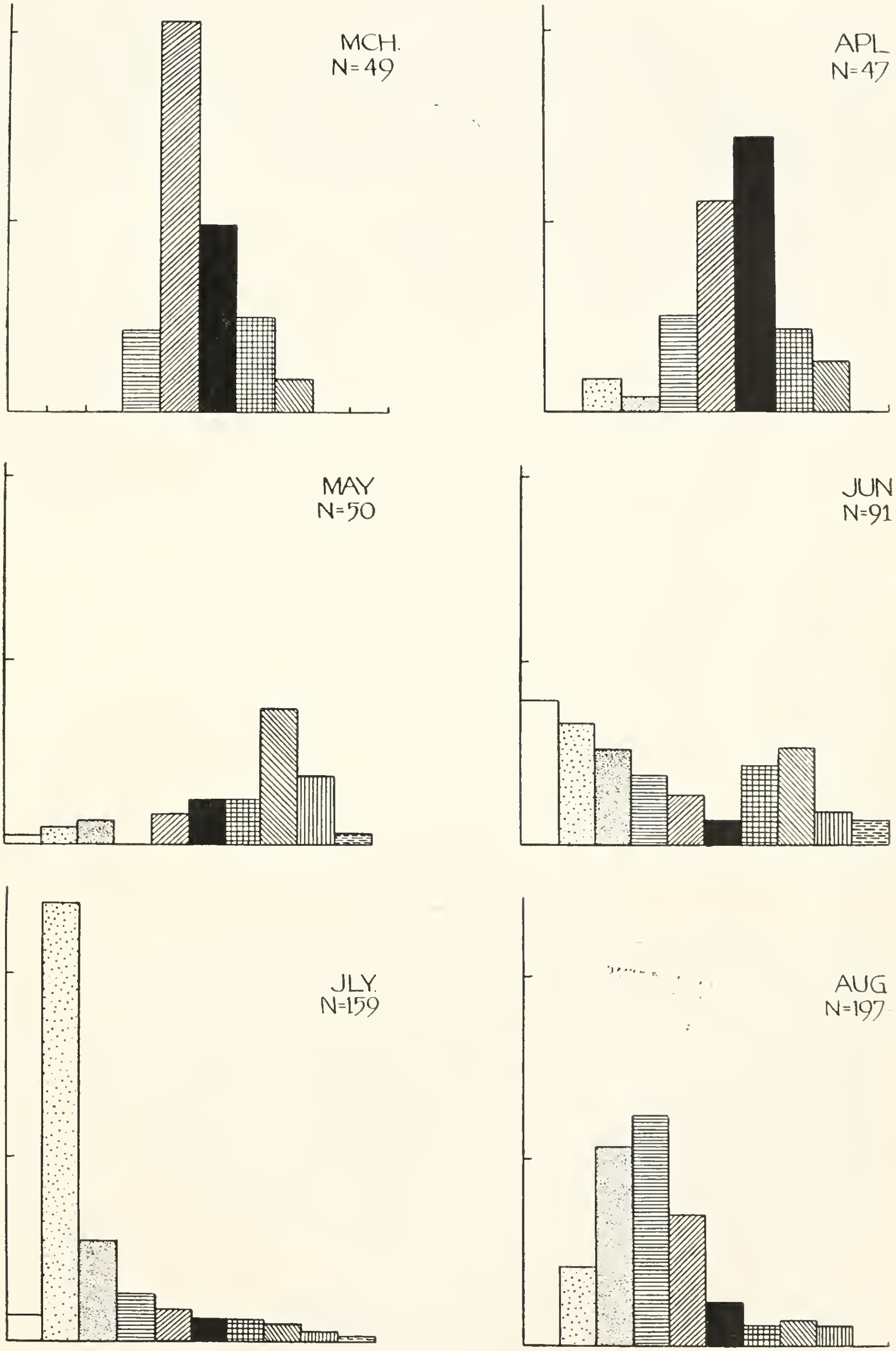

FIGURE 3.—*Continued.*

GROWTH OF INDIVIDUALS

The mean length of the individuals in each sample was calculated from the equation $M = \frac{\Sigma fX}{N}$, where N = the total number of individuals measured, f = the total number of individuals in each size group, and X = the mid-point of each size group.

The results are arranged in Table II, while the monthly variation of arithmetic mean length is shown in Figure 2. It will be seen that the mean length increased from 8.25 cm. towards the end of September, to 11.29 cm. in November. Throughout the winter from November to February the mean length decreased, reaching a minimum in late February. This fall may be explained by the elimination of the worms falling into the larger size groups, but it may also be explained as due to a decline in length of the larger worms owing to the partial resorption of their own tissues, or to the maturation of the coelomic oocytes which may prevent complete relaxation of the body when narcotized. This fall during winter is statistically significant and is unlikely to have been due to inaccurate or insufficient sampling. The mean length increased steadily during the spring, unspawned survivors of the old generation having grown rapidly. From May to July the mean length again fell, owing not only to the partial elimination of the unspawned worms of the old generation, but mainly to the inclusion of the young worms resulting from the spawning during the previous February. Thereafter, the mean length increased from July until the onset of winter.

TABLE II

Monthly variation of total length

Month	Size-group (cm.)										N	M
	0–2	2–4	4–6	6–8	8–10	10–12	12–14	14–16	16–18	18–20		
1948												
September (29:9:48)	—	1	6	11	40	3	—	—	—	—	61	8.25
October	—	2	9	12	21	17	8	—	—	—	69	8.92
November	—	—	4	3	15	34	33	7	—	—	96	11.29
December	—	—	2	2	9	10	9	1	—	—	33	10.52
1949												
January	—	—	1	3	9	14	2	—	—	—	29	9.99
February	—	—	2	10	25	11	4	—	—	—	52	9.19
March	—	—	—	5	25	12	5	2	—	—	49	10.42
April	—	2	1	6	13	17	5	3	—	—	47	9.94
May	1	2	3	0	4	6	6	18	9	1	50	12.98
June	18	15	12	9	6	3	10	11	4	3	91	7.78
July	6	95	22	10	7	5	5	4	3	2	159	5.44
August	—	21	53	61	35	11	5	6	5	—	197	7.27
September	—	9	17	46	76	41	27	12	8	3	239	9.60

It will be apparent that the worms steadily increased in length during the first summer after hatching (Fig. 4). The rate of growth of larvae and young worms during the first two months has already been described (Dales, 1950). The rate of growth during later life may be inferred from the monthly means, but this is not possible during mid-summer, since the frequency distribution of the individuals when plotted against length is bimodal owing to the presence of two sharply distinct generations. In these instances, the values of the two component means can only be judged by inspection. There seems to have been little or no increase in length during winter, but during summer growth of unspawned females of the old generation—that is, worms nearing 18 months in age—was resumed, some of these worms eventually attaining a length of 20 cm. It is unlikely that any of these latter worms survived the following winter. However, some females which for some reason were slow in maturing, and were undersized when a year old may possibly have lived to spawn (for the first time) in their second year.

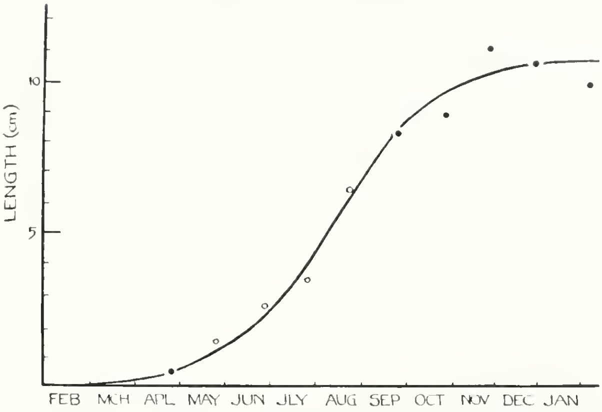

FIGURE 4. Growth of young worms during the first year. Empty circles represent estimated values; filled circles values calculated directly from the data.

CONCLUSIONS

It may be concluded that in this population at least, the length of life does not normally exceed 18 months, and includes only one breeding season. During summer worms grew rapidly, increasing 1.0–2.0 cm. in length each month, young worms hatched in February reaching a length of 10 cm. by October. There was virtually no increase in length during winter, but the germ cells grew rapidly during this period, spawning taking place in early spring. Spawned worms soon died, but owing to the comparative rarity of the males and the fact that the females will not spawn except in the presence of a male, large numbers of mature females were, in effect, surplus, and remained unspawned. These eventually died before the following spawning season, but many attained a relatively large size before so doing.

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EFFECT OF X-RADIATION ON THE DESOXYRIBONUCLEIC ACID AND ON THE SIZE OF GRASSHOPPER EMBRYONIC NUCLEI [1, 2]

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It is now believed that the vital activities of cells are controlled to a great extent by nucleoproteins. Only a beginning has been made toward attaining an understanding of the biological importance of nucleic acids. Research methods which involve the use of agents that disturb the normal balance of the nucleic acids are being utilized, and by these means it should be possible to reach some definite conclusions on the role played by the nucleic acids in living cells.

Since desoxyribonucleic acid (DNA) is a universal constituent of chromatin, it probably has a significant part in inheritance, in regulating cell division, and perhaps in the formation of proteins and of ribonucleic acids of the nucleolus and cytoplasm (Caspersson, 1936, 1940, 1941; Caspersson and Schultz, 1938, 1940). Since x-rays produce mutations and chromosomal aberrations, observations on their effect on the DNA content of the cells are of considerable importance. We had previously noted in this laboratory that irradiated nuclei seem to stain less intensively with the Feulgen reagent than non-irradiated nuclei. The purpose of the present study was to determine by photometric methods, which make possible an accurate quantitative cytochemical study of the nucleoproteins, whether there is an actual change in the quantity of DNA after irradiation. Examination of our photometric data sheets disclosed that the diameters of irradiated nuclei were usually larger than those of control nuclei. This fact, along with an apparent increase in size observed in irradiated neuroblasts in living culture preparations, led to a study of the effect of radiation on nuclear size.

Methyl green stain and the Feulgen reaction were used for the DNA determinations. With these two stains it is possible to analyze for two different properties of the nucleic acid. The Feulgen is specific for the DNA desoxypentose content and the methyl green probably indicates the presence of DNA in the normal state of polymerization, commonly called the "highly polymerized state." Most workers now agree that, when the nuclear reaction takes place under properly controlled conditions, the Feulgen method gives an accurate relative estimate of the DNA in nuclei (Hillary, 1940; Baker, 1942; Stowell, 1945, 1946; Di Stefano, 1948). Ultraviolet absorption studies of Wyckoff, Ebeling and Ter Louw (1932) identify the Feulgen-positive material in the nucleus with DNA. Also, splenic and pancreatic desoxy-

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ribonucleases remove the Feulgen-positive bands (presumed to contain large amounts of DNA) from the salivary gland chromosomes of Drosophila (Catcheside and Holmes, 1947). Although there is evidence that the reaction does not indicate the total amount of DNA present (Caspersson, 1932; Ely and Ross, 1949), many workers have shown it is valid to use the Feulgen reaction with the photometric system to determine the relative amounts of DNA in nuclei (Ris and Mirsky, 1949; Lumb, 1950; Leuchtenberger, 1950; Swift, 1950). The specificity of methyl green for polymerized DNA was demonstrated by Pollister and Leuchtenberger (1949), Leuchtenberger (1950), and Kurnick (1950a, 1950b, 1950c). Leuchtenberger found Feulgen and methyl green extinction ratios to be equal in normal nuclei.

MATERIAL AND METHODS

The present cytochemical studies were made with 14-day-old embryos of the grasshopper, *Chortophaga viridifasciata* (De Geer). The control and irradiated embryos for each experiment were from the same egg case. Doses of 4000, 10,000, and 12,500 r of x-rays were given. The embryos were fixed 10 hours after irradiation, since the maximum visible difference between the irradiated and control tissue, along with the maximum swelling of the irradiated nuclei, was observable at this time (Figs. 1–3). Both control and irradiated eggs were kept at 38° C. between the time of irradiation and fixation. Treatment of the control and x-rayed embryos was identical throughout each experiment except for the irradiation, thereby ruling out any disparity of the results from other causes and making valid a comparison of the cells of the irradiated and control embryos.

Since many cytologists (Koller, 1947; Darlington and Mather, 1949; Serra, 1949; etc.) believe that the content of DNA per nucleus varies with the different stages of mitosis, all photometric measurements were made on cells in the mitotic stages in which the chromatin is most widely dispersed, namely, late telophase, interphase, and early prophase. All three of these stages were used because it was difficult to distinguish them from one another in material which had been treated by immersion in approximately 20 per cent of the isotonic salt concentration to make the chromatin more uniformly distributed throughout the nuclei.

Two different x-ray units were used in these studies. The first experiments were done with a Coolidge Universal x-ray tube, operating at 90 kilovolts and 5 milliamperes. During exposure the material was 18.4 cm. from the center of the target, and the long wave length x-rays were removed by a 0.3 mm. aluminum filter. The intensity was about 250 r per minute. Later, when the previous experiments were repeated and additional studies made, irradiation was carried out with a General Electric Maximar 250 kv unit operating at 250 kilovolts and 15 milliamperes. An aluminum filter of 0.5 mm. was added to the 3 mm. aluminum inherent filtration of the machine. When the material was 54 cm. from the center of the target, the intensity was about 250 r per minute. Monitoring with a Victoreen thimble chamber preceded each x-ray treatment.

All material to be sectioned was fixed with Carothers fluid.[5] The fixation time

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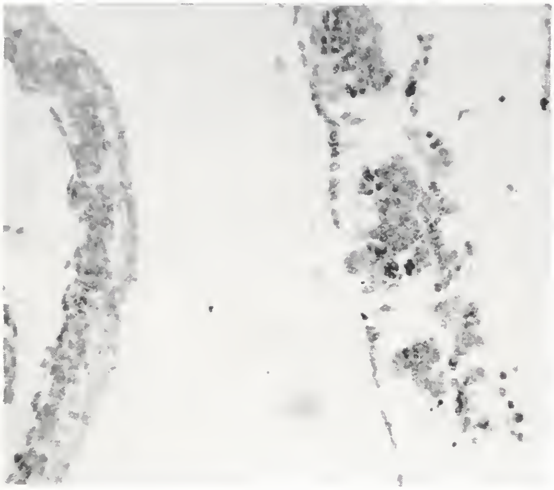
[5] 75 cc. of a saturated aqueous solution of picric acid; 15 cc. of formalin, C. P.; 10 cc. of glacial acetic acid; 1 gm. urea crystals. Warm slightly and stir thoroughly as the urea is added. Add 4 drops of a 50 per cent aqueous solution of chromic acid to 5 cc. of the above solution just before using.



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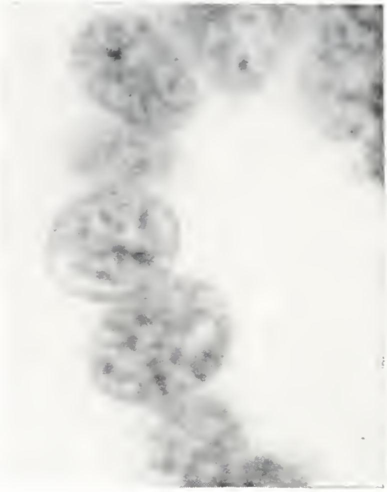

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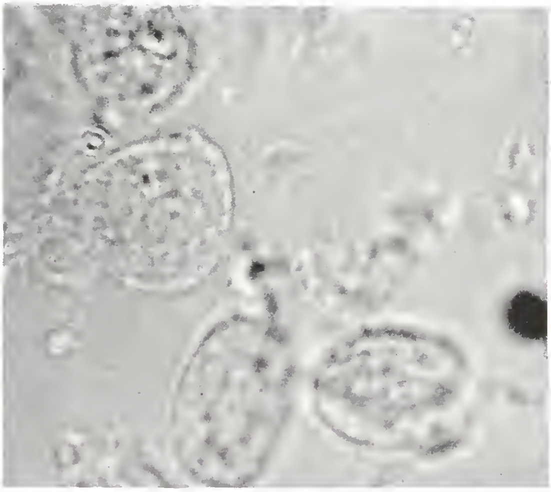

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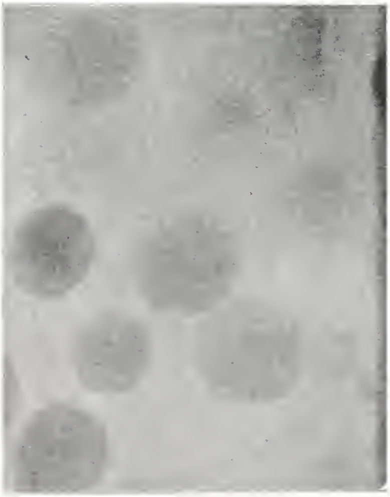

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FIGURE 1. Note the difference in Feulgen stainability between the irradiated embryo (4000 r) on the left and the non-irradiated embryo on the right. Fixed with Carothers fluid 10 hours after treatment. 100 ×.

FIGURE 2. Heterogeneous un-irradiated nuclei. Carothers fixation. Feulgen stained. 900 ×.

FIGURE 3. Heterogeneous irradiated nuclei. Fixed with 4 per cent neutral formalin, stained with methyl green. Note the enlarged nuclei. 900 ×.

FIGURE 4. Un-irradiated nuclei pre-treated with a hypotonic solution that made the chromatin more uniformly distributed. Fixed in 4 per cent neutral formalin, stained with methyl green. 900 ×.

of the control and treated embryos for each experiment was the same, varying from 20 to 24 hours for the different experiments. The embryos were washed in distilled water for one hour and then prepared for sectioning by dehydrating with ethanol. The picric acid of the fixative was removed in a 70 per cent ethanol solution saturated with lithium carbonate. After dehydration was complete, the material was cleared in aniline oil and chloroform and embedded in Tissuemat. The control embryo and treated embryo were embedded side by side in the paraffin block and sectioned simultaneously. Sections were cut at 16 microns, then decerated and hydrated. A hydrolysis curve for each time was run in normal HCl (58° C.) for 10, 20, 30, and 40 minutes. The optimum Feulgen staining reaction, which was used for photometric determinations, was obtained after 20-minute hydrolysis. The Feulgen stain was prepared as specified by Coleman (1938). With the exception of the staining time, 3 hours for these sections, the staining schedule of Darlington and LaCour (1947) was followed.

Embryos dissected in a hypotonic solution and then fixed in acetic-alcohol or neutral formalin have nuclei which show a more uniform distribution of chromatin than cells dissected in an isotonic solution (Figs. 2, 3, 4). Variations in the absorption measurements of a nucleus were reduced by making the chromatin more uniformly distributed. Smears of the more uniform-appearing nuclei were stained with Feulgen and methyl green in the following manner. Embryos were removed from the eggs and dissected in a hypotonic culture medium and left in the solution for 10 minutes. Cells that were to be stained with the Feulgen reagent were fixed in Carnoy fluid (3 : 1) for 30 minutes. After hydrolysis for 10 minutes in normal HCl at 60° C., the embryos were rinsed in distilled water, stained with leucobasic fuchsin for 15 minutes, softened for smearing by treating with 45 per cent acetic acid for 1 minute, and then mounted and smeared in a Karo-formalin solution (1 part Karo, 1 part formalin, 3 parts water). The preparation and methyl green staining procedure described by Kurnick (1950a) was followed, with the exceptions that the embryos were dissected in a hypotonic culture medium before fixation and that the staining was done at 17° C. Cells treated in the same way except for staining were used to determine the blank, *i.e.*, the absorption of unstained nuclei. Correction for nonspecific light loss was not made after it was shown that unstained nuclei are practically invisible and transmit from 93 to 96 per cent of the light.

Measurements of nuclear diameters were made with an oil immersion objective (90 ×) and a 10 × ocular which contained a calibrated micrometer disk. Since all the nuclei were not exactly spherical, four different diameters of each nucleus were read and averaged. The mean of ten such averages, all on nuclei from the same embryo, was obtained for each dose of x-rays and for the corresponding controls. Over 300 nuclei were measured. An example of the data is shown in Table I.

The photometric apparatus used is similar to that described by Swift (1950) with the micro-ammeter replaced by an amplifier and Brown recorder. The recorder was originally installed to facilitate some tests of the apparatus, and since it offered the advantage of an automatic permanent record of the photoelectric current, it was not removed from the system. The DNA stained with the Feulgen reagent was measured by the absorption of green light isolated from a Spencer microscope lamp by means of Corning Glass filters No. 3484, No. 4010, and No. 4303. The DNA stained with methyl green was measured in terms of the absorption of red light isolated by Corning Glass filter No. 2408. Optics were an A. O. 50 × reflecting objective and a Zeiss 10 × ocular.

DNA content is expressed in relative instead of absolute units, because several assumptions are made with photometric microscopic measurements, namely, that the nuclei are perfect spheres, and that the material is homogeneously distributed throughout the nucleus. With Feulgen-stained material variations in fixation and staining procedure may produce variations in the intensity of the reaction. Also, potentially stainable material may be lost during hydrolysis. In view of these assumptions and possible sources of error, it is not feasible to determine absolute amounts of DNA per nucleus. All values of DNA reported are, therefore, in arbitrary units.

Table I

Data showing measurements of diameters of control and irradiated (4000 r) nuclei. Sections fixed with Carothers fluid, hydrolyzed 20 minutes, and Feulgen-stained

Embryo	Nuclei	Diameter measurements				Average	Mean
		1	2	3	4		
Irradiated (4000 r)	1	10.5	10.0	11.0	10.5	10.50	
	2	10.5	10.0	8.5	8.0	9.25	
	3	9.0	9.0	8.5	8.0	8.62	
	4	8.5	8.5	7.5	9.0	8.37	
	5	10.5	10.0	11.0	11.0	10.62	
	6	8.0	8.5	8.5	8.0	8.25	
	7	8.5	10.5	10.0	10.5	9.87	
	8	9.0	10.0	9.0	11.0	9.75	
	9	8.0	9.0	8.0	9.0	8.50	
	10	9.0	8.5	8.5	8.5	8.62	
							9.24
Control	1	8.5	10.0	10.0	10.5	9.75	
	2	7.5	8.0	8.5	7.5	7.87	
	3	7.5	8.0	8.5	7.5	7.87	
	4	6.5	7.5	7.5	7.5	7.25	
	5	8.5	8.0	6.0	7.5	7.50	
	6	8.0	8.5	8.0	8.0	8.12	
	7	9.0	9.0	8.0	9.0	8.75	
	8	8.5	8.0	10.0	9.0	8.87	
	9	9.0	8.5	9.0	10.0	9.12	
	10	9.0	8.5	9.0	10.5	9.25	
							8.43

A brief explanation of the photometric routine will aid in understanding the tables. A nucleus which is to be measured is centered in the microscope field and brought into sharp focus. The photometric apparatus is adjusted over the ocular, and an enlarged image of the nucleus is observed through a magnifying lens placed immediately above the field diaphragm of the photometer. Measurements of the nuclear diameter are made, after the nucleus has been made concentric with the micrometer disk in the image plane. An aperture of 1, 2, or 3 millimeter diameter is inserted in the image plane, so that only a central core of the nucleus is visible. The magnifier is then swung out, and the phototube is moved into place over the aperture. Light from the central core of the nucleus now falls on the photosensitive cathode, and a reading is made with the Brown recorder. Immediately the slide is moved and a clear area of the slide is measured in a similar manner. Two readings, the first through the nucleus and the second through the background, are made for each determination. The intensity of the light passing through the clear area (I_0) divided by the intensity of the light transmitted by the nucleus (I_n) gives the reciprocal

TABLE II

Samples of raw data, showing the calculations made in obtaining the arbitrary units given in subsequent tables

Nuclear diameter* (μ)	I_n	I_0	I_0/I_n (1/Transmission)	Extinction (log $1/T$)	$1/F$	EC^2 ($1/F$)
Nuclei irradiated 4000 r. Carothers fixation. Feulgen sections.						
9.2	59.1	93.2	1.577			
	59.6	92.7	1.555			
	59.6	92.4	1.550			
	60.7	91.7	1.511			
	58.8	92.4	1.571			
			1.553	0.19117	6.476	2.78
9.0	53.7	91.8	1.709			
	52.8	93.1	1.763			
	53.3	91.1	1.709			
	54.8	91.6	1.671			
	52.5	90.6	1.726			
			1.716	0.23452	6.115	3.23
Non-irradiated (control) nuclei. Carothers fixation. Feulgen sections.						
6.5	40.8	91.8	2.250			
	40.4	90.9	2.250			
	40.5	92.4	2.281			
	40.4	90.7	2.245			
	40.2	90.3	2.246			
			2.254	0.35295	3.29	2.61
6.2	30.1	86.6	2.877			
	29.1	87.1	2.993			
	29.0	87.6	3.021			
	29.8	87.2	2.926			
	30.6	87.4	2.856			
			2.935	0.46761	2.99	3.15

* An average of five measurements.

of the transmission ($1/T$). Transmission data are then converted into extinction values (E):

$$E = \text{Log}_{10}\, 1/T.$$

This extinction value represents the light transmission for the central core of the nucleus and not through the entire nucleus. A value which gives the relationship of the area measured to the entire volume of the cell is $C^2\, 1/F$.

$$1/F = \frac{R^3}{R^3 - (R^2 - C^2)^{3/2}}$$

C is the radius of the cylinder or the radius of the aperture used to make the reading. R is the radius of the spherical nucleus. Then $EC^2 1/F =$ extinction value for the total volume of the nucleus (Table II). Further details of the computations and corrections for photometric measurements are discussed by Swift (1950), Pollister and Ris (1947), and Di Stefano (1948).

STATISTICAL TREATMENT

To ascertain whether a significant difference existed between the mean diameters of irradiated and control nuclei, a test devised by Mosteller (1948) was used. The 2.0 per cent level was accepted as significant.

A two-way analysis of variance table was used to determine the difference in DNA (Feulgen and methyl green) content of irradiated and control nuclei. Unless stated otherwise, the 5 per cent probability level was accepted as the significant value.

RESULTS

Nuclear diameter. When studying a series of Feulgen-stained nuclei of any one type, the fact becomes apparent that smaller nuclei are more darkly stained than larger nuclei. This condition was also noted by Swift (1950). Measurements of the diameters of nuclei of the Feulgen and methyl green material showed that the irradiated nuclei had significantly larger diameters than the non-irradiated (Tables III

TABLE III

Data showing mean diameters of control and irradiated nuclei. Feulgen-stained section; Carothers fixation

Treatment	Hydrolysis time (min.)	Number of nuclei measured	Mean diameter (μ)
X-ray 4000 r	10	20	11.55
Control	10	20	10.29
X-ray 4000 r	20	40	11.85
Control	20	40	10.85
X-ray 4000 r	30	20	11.70
Control	30	20	10.22
X-ray 4000 r	40	20	11.50
Control	40	20	10.42

TABLE IV

Data showing mean diameters of control and irradiated nuclei. Methyl green smears; fixation in 4 per cent neutral formalin

Treatment	Number of nuclei measured	Mean diameter (μ)
X-ray 4000 r	30	10.13
Control	30	8.26
X-ray 10,000 r	20	11.15
Control	20	9.47

and IV). Nuclei that had received an x-ray dose of 12,500 r were not significantly larger than nuclei receiving only 4000 r. Therefore, the degree of swelling at 10 hours after treatment does not appear to be related to the dose of irradiation received. Living-culture experiments performed in this laboratory have shown that the neuroblasts of grasshopper embryos swell after receiving any dose greater than 100 r of gamma or x-radiation. This swelling is independent of the dose rate, as it was observed whether a dose of 100 r was given in 1 minute or in six days. A change in osmotic pressure accompanies the swelling of cells, since after irradiation the cells are hypertonic in a culture medium in which they were isotonic previous to irradiation.

TABLE V

Average amounts of DNA per nucleus in embryonic grasshopper tissue, as obtained by photometric determinations of Feulgen preparations *

	Arbitrary units of DNA			
	Carothers fixation (sectioned material)		Carnoy fixation (smears)	
	Control	Irradiated 4000 r	Control	Irradiated 12,500 r
	3.18	2.78	3.51	4.93
	2.80	3.23	2.99	4.99
	2.61	2.61	3.55	4.90
	6.22†	2.34	3.72	3.94
	3.15	2.19	2.86	2.48
	3.05	2.83	3.15	3.02
	3.16	2.89	3.27	3.44
	3.32	2.20	3.62	2.96
	2.91	3.32	3.92	3.28
	2.96	2.38	2.72	4.83
	3.00	2.87	3.08	4.52
	2.69	3.00	2.89	4.86
	2.73	3.23	3.59	2.83
	3.42	3.18	2.27	3.84
	3.23	2.22	3.42	3.69
	2.69			
Mean	2.99	2.75	3.24	3.90
Standard error	0.28	0.26	0.31	0.40

* Each value is an average of five readings on one nucleus.

† Not averaged with other values since this cell probably had 4N DNA content.

Feulgen determinations. Photometric measurements on tissue stained with Feulgen show, when corrections for nuclear volume are made, that approximately the same number of dye molecules exist in irradiated and non-irradiated nuclei. Statistically, there was no significant difference between the irradiated and control cells (Table V).

Methyl green determinations. Photometric data on methyl green stained material revealed a highly significant difference (1 per cent level) between the extinction values of the irradiated (4000 r and 10,000 r) and non-irradiated nuclei. The results are summarized in Table VI.

TABLE VI

*Average amounts of DNA per nucleus in embryonic grasshopper tissue, as obtained by photometric determinations of methyl green preparations **

	Arbitrary units of DNA			
	Control	Irradiated 4000 r	Control	Irradiated 10,000 r
	6.34	3.11	4.45	2.66
	5.92	2.44	2.29	1.90
	4.23	2.13	5.66	1.89
	4.86	1.79	6.74	1.45
	4.57	1.85	3.48	0.88
	6.02	2.55	5.83	1.46
	5.36	2.02	5.22	1.78
	5.86	2.43	3.46	1.83
	5.55	3.01	2.99	1.48
	6.00	3.02	6.42	1.96
	4.23	1.87	3.25	1.83
	4.86	1.58	3.48	2.02
	4.43	2.31	2.92	2.42
	5.57	2.46	4.54	2.23
	4.69	2.98	6.83	2.41
	4.36	2.72	6.47	1.87
	4.15	2.83	5.25	2.36
	6.55	1.89	5.83	1.84
Mean	5.20	2.39	4.73	1.90
Standard error	0.19	0.11	0.35	0.10

* Each value is an average of five readings on one nucleus.

With nuclei which had been made fairly homogeneous, it was difficult to distinguish between late telophase, interphase, and early prophase. Photometric measurements disclosed a few nuclei which had twice the DNA content of the other nuclei (Table V). The increased amount of DNA may be attributed to a polyploid nucleus resulting from a radiation-induced suppression of cytokinesis.

DISCUSSION

The present study has shown that an increase in nuclear size occurs after x-radiation. This has been observed in a wide variety of biological materials. Holweck and Lacassagne (1930) found that yeast spores divided and then swelled after irradiation. Similar results were obtained by Robertson (1935) with protozoa; Lea, Haines and Coulson (1937) with bacteria; Mottram (1927) with mice tumors; Glücksmann (1941) with human tumors. Lea (1947) explained such cases, where the swelling did not appear for several hours or days after treatment, by stating that the effect was due to an inhibition of cellular division while growth continued at a normal rate. If our studies had been based entirely on stained material (fixed 10 hours after irradiation), Lea's explanation would have satisfied the data. However, our experiments with living tissue cultures have shown that swelling occurs in a matter of minutes after treatment. When the larger doses are given, the swelling is instantaneous. A normal growth rate can hardly account for such rapid swelling. Observations on irradiated tissue cultures lead us to believe that water is being absorbed rapidly by the cells, presumably as a result of an x-ray-induced osmotic pressure increase. Failla (1940) suggested that radiation might change the cellular constituents so that the osmotic pressure is increased without altering the permeability of the cellular membrane. The depolymerization or molecular rearrangements of DNA, indicated by the methyl green results, might then be a factor contributing to the increase of osmotic pressure and thus cause the nuclei to swell.

Probably the first study reporting that radiation could increase the nuclear volume without decreasing the amount of chromatin was made by Mottram in 1933 on work with bean root tips. After accurate and detailed visual observations he wrote (p. 218) that the increase in size of the nuclei after radiation "is due to a great increase in the nucleoplasm, the other constituents of the nucleus remaining normal in amount. The chromatin becomes thus more dispersed and has the appearance of being diminished in amount, but in reality it remains normal in quantity." Results suggesting that the nuclear volume may be changed considerably without a significant variation in the Feulgen-stainability of DNA, are in agreement with recent work by Schrader and Leuchtenberger (1950), and Leuchtenberger (1950). The former authors reported that the DNA per nucleus remained constant, despite an eight-fold increase of nuclear volume in the spermatocytes of *Arvelius albopunctatus*. Additional studies disclosed that an increase or decrease of nuclear volume is due mainly to a corresponding modification in the protein, RNA, and water content of the cell and not to an alteration of DNA. Leuchtenberger found in early pycnosis of tumor cells, a condition in which marked reduction of nuclear volume occurred, the Feulgen-stainability of DNA remaining unchanged in quantity. The conclusion seems justified that x-radiation may augment the nuclear volume without destroying the DNA desoxypentose.

The cytochemical studies using methyl green show that some physical property of DNA is affected. Probably depolymerization or some change of the molecular configuration has taken place, and the change is too slight to detect visually. Many investigators report that depolymerization of the DNA may occur after treatment with ionizing radiations. In 1939 Svedberg and Brohult suggested that, directly or indirectly, radiation splits the molecular constituents within the cell. The stickiness and loss of spiralization noted in chromosomes after irradiation may be produced by the depolymerization of nucleic acids (Darlington and La Cour, 1945). Sparrow and Rosenfeld (1946), and Taylor, Greenstein and Hollaender (1947) report x-ray effects on isolated DNA but conclude that the nucleic acid is not destroyed, since no chemical property is altered. Both investigations reveal a loss of streaming birefringence and a great drop in viscosity of the DNA after x-ray treatment. Since both the magnitude of relative viscosity and the intensity of flow birefringence are indicative of the degree of molecular asymmetry, the above changes in these properties probably represent a depolymerization or rearrangement of the molecular particles.

Direct observations with a microscope of Feulgen-stained tissue show that nuclei which were given a dose of 4000 r or more are less intensively stained than non-irradiated nuclei. However, when nuclear measurements and light absorption readings are made, it is evident that there is not a significant decrease of DNA desoxypentose. The apparent visual loss of Feulgen stainability would seem to be the result of nuclear swelling, that is, a change in the distribution of the DNA in the nucleus. With microscopic observations the intensity and color of irradiated and control nuclei appear to be the same in tissue stained with methyl green, but photometric measurements disclose a highly significant loss in stainability after radiation.

We are very appreciative of the counsel and encouragement given us by Dr. J. Gordon Carlson of The University of Tennessee and Dr. Mary Esther Gaulden of the Oak Ridge National Laboratory. We gratefully acknowledge our indebtedness to Mr. Jack Moshman of the Oak Ridge National Laboratory Mathematics Panel for the statistical treatment of our data.

SUMMARY

1. Cytological and cytochemical studies were made on embryonic nuclei of the grasshopper, *Chortophaga viridifasciata*, after x-ray doses of 4000, 10,000, and 12,500 r. The changes induced were photometrically measured by using (1) the Feulgen reaction to determine relative changes in the DNA desoxypentose, and (2) the methyl green stain to indicate the degree of polymerization of the nucleic acid.

2. X-radiation was found to cause swelling of the nuclei. When correction was made for this, the Feulgen-stained nuclei showed no significant loss of DNA after irradiation, but the nuclei stained with methyl green disclosed a highly significant loss of stainability. This is interpreted to indicate that x-rays do not destroy the DNA but induce depolymerization of the nucleic acid.

3. This study has shown that it is not safe to make quantitative estimates of DNA (stained by Feulgen or methyl green) from microscopic observations and photographs. The visually apparent loss of Feulgen-stainability after irradiation is probably not due to a decrease in the DNA desoxypentose but rather to the increased dispersion of the DNA in nuclei that have undergone x-ray-induced enlargement.

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EXISTENCE OF PHYSIOLOGICALLY-DIFFERENT RACES OF OYSTERS, CRASSOSTREA VIRGINICA

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Until recently it was generally accepted that the population of the American oyster, *Crassostrea virginica* Gmelin, which extends along the Atlantic Coast from Prince Edward Island, Canada to Florida, and inhabits the Gulf of Mexico, was composed of individuals of the same physiological race, or variety, showing the same physiological requirements, such as the lowest temperature at which they could begin spawning. This opinion was especially well crystalized by Nelson (1928a) in his discussion of the so-called "critical temperature for spawning of oysters," where he concluded that our oysters, as well as other lamellibranch mollusks, begin to breed at a definite temperature which is constant for the species. Somewhat later he (Nelson, 1928b) expresses this view even more precisely by stating (p. 154) that "The American oyster *Ostrea virginica* Gmelin spawns after the water temperature reaches 20° C., over all parts of its range, with no adjustment to the extremes of its distribution."

Nelson's opinion, with which most of the oyster biologists of that period agreed, prevailed uncontested until Hopkins (1931) and Loosanoff (1932) working at Galveston Bay, Texas, and James River, Va., respectively, showed that in those areas the temperature of 20.0° C. was reached far ahead of the ripening of gonads of oysters. Therefore, spawning of oysters there began at a considerably higher temperature, usually when it was near 25.0° C.

A further and, perhaps, decisive argument against the theory was raised when it was found that some of the oysters of Long Island Sound were spawning at temperatures ranging between 16.4 and 18.5° C. (Loosanoff, 1939). Thus, contrary to the established theory, it became clear that not all local groups of *C. virginica* of the range occupied by this species begin to spawn when the temperature of 20.0° C. is reached. Actually, different groups appeared to require different temperatures. This suggested that the oysters composing the different geographical groups may belong to physiologically-different races.

As far as we could establish, the first reference in the literature to the possibility of the existence of physiologically-different races within the population of the same species, *C. virginica*, was made by Loosanoff and Engle (1942). The suggestion was based upon the differences in the spawning behavior shown by the different groups of the oyster population of Long Island Sound. Several years later Loosanoff and Tommers (1948), while discussing the behavior of oysters in turbid waters, again mentioned that there appear to be physiologically-different races within the general population of *C. virginica*, some of which have developed a greater tolerance to turbidity than the oysters of Long Island Sound. Recently Stauber (1950) reviewed the rather limited material pertaining to the possibility of existence of physiologically-different races of *C. virginica*, emphasizing the need for additional research in this field. The present article is our preliminary contribution to the solution of this interesting problem.

The first steps to determine experimentally whether oysters of different geographical areas show different physiological traits were taken in the fall of 1948. Groups of young oysters, which had metamorphosed that summer, were received from Warren, Mass., Cape May, N. J., Seaside of Va., James River, Va., Pamlico Sound, N. C. and Pensacola, Fla. They were suspended in large wire trays under identical conditions in Milford Harbor. A group of Long Island Sound oysters of the same age was placed under the same conditions to serve as the control.

The chief trait to be studied was the ability of the oysters of different geographical areas to discharge spawn in our waters, where the maximum water temperatures reached during the summers are usually considerably lower than those of more southern areas. Furthermore, in Long Island Sound the period of relatively high temperatures, 20.0° and above, is of rather short duration, seldom exceeding 8 or 9 weeks (Loosanoff, 1937), while in James River, Va., from where one of our southern samples came, such temperatures may persist from about May 10 to about October 20, a period of 160 days, or about 23 weeks (Loosanoff, 1932).

Observations on the North Carolina and Florida oysters were discontinued in the spring of 1949 because 87 per cent of the first, and 97 per cent of the second group died during the preceding winter. Death was tentatively ascribed to the prolonged low temperature under which the oysters remained, but the final conclusion regarding this matter will be formed only after additional observations on the survival of southern oysters in our waters are completed.

Detailed gross and histological examinations of the remaining oysters were made on September 27, 1950. Samples, each consisting of 20 specimens, were taken from each group. By that time, all the oysters involved in these studies had spent approximately two years in Milford Harbor and, thus, were considered as having fully recovered from any detrimental conditions that might have accompanied the shipping of the oysters from their place of origin to Milford. At the time of collection the temperature of the water was already down to 16.0° C.

The results showed clearly that the gonads of the oysters of the different groups were in decidedly different conditions (Table I). All the individuals comprising the two northern groups, representing the Massachusetts and Long Island Sound populations of oysters, were completely spawned out (Fig. 1). In most instances the gonads had already passed through the stage of resorption of pycnotic cells left after spawning, and the oysters were accumulating glycogen, a normal process closely correlated in local waters with seasonal gonadal changes (Loosanoff, 1942).

In contrast with the conditions found in northern oysters, the majority of those of the three southern groups still retained all or the largest part of their spawn (Table I and Fig. 2). A few, nevertheless, possessed completely or almost completely empty gonads, although it was not determined, with absolute certainty, whether those oysters were spawned out or their gonads were resorbed after failure to spawn.

In all three southern groups many oysters had creamy, soft gonads, the thickness of which was often as much as 3.0 mm. However, in the other oysters of the same groups the consistency of the gonadal layer was, regardless of its thickness, somewhat harder than usual indicating, perhaps, that cytolysis and resorption of the undischarged material was already in progress. This condition was even more pronounced in the oysters with partially full gonads, which, as a rule, were characterized by the presence of extremely large numbers of phagocytic cells. However, even in those oysters some of the spermatozoa and eggs were normal in appearance, the spermatozoa usually becoming active if placed in a drop of water.

The average thickness of the gonadal layer of the oysters of the three groups ranged from 1.2 to 2.3 mm., being the thickest in the James River, Va. lot which was the most southern group (Table I).

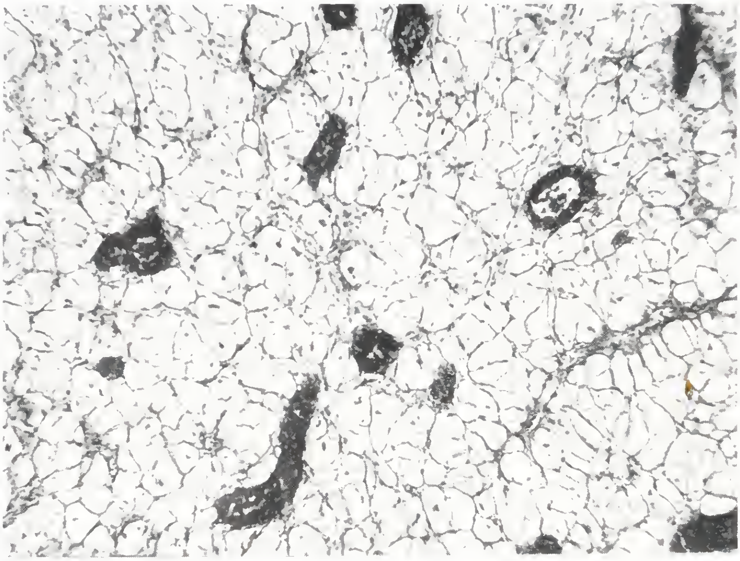
Attempts to induce spawning of the oysters of the three southern groups failed. None of the oysters released any spawn despite the fact that a combination of temperature and chemical stimuli was tried. The stimulated oysters moved their shells as in spawning but, nevertheless, released no eggs or sperm. It is possible that at that time the degenerative processes within the gonads had already progressed to such an extent that normal spawning became impossible.

TABLE I

Conditions of gonads of oysters originated in different geographical areas, kept in Milford Harbor, Conn. for about 2 years. End of spawning season, 1950

Area of origin	Conditions of gonads			Gonad thickness, range in millimeters	Average gonad thickness in millimeters
	Unspawned	Partly empty	Empty		
		September 27 (20 oysters per sample)			
Wareham, Mass.	0	0	20	0.0	0.0
Long Island Sound	0	0	20	0.0	0.0
Cape May, N. J.	7	12	1	0.0–3.0	1.2
Seaside, Va.	10	8	2	0.0–3.0	1.6
James River, Va.	15	3	2	0.0–3.5	2.3
		October 25 (10 oysters per sample)			
Wareham, Mass.	0	0	10	0.0	0.0
Long Island Sound	0	0	10	0.0	0.0
Cape May, N. J.	2	3	5	0.0–3.0	0.6
Seaside, Va.	4	5	1	0.0–3.5	1.7
James River, Va.	4	6	0	1.0–3.0	2.1

The second examination was made on October 25, 1950 when the temperature of the water in Milford Harbor had decreased to 14.0° C. On that occasion, however, only 10 oysters per sample were taken. The examination again emphasized the differences between the northern and southern groups. The two northern groups were, of course, again found with discharged gonads and in the process of accumulation of glycogen. The southern groups, however, still contained a considerable quantity of spawn (Table I). In the Cape May, N. J. group, half the oysters contained all or some spawn. Active sperm could still be found but eggs, as a rule, although



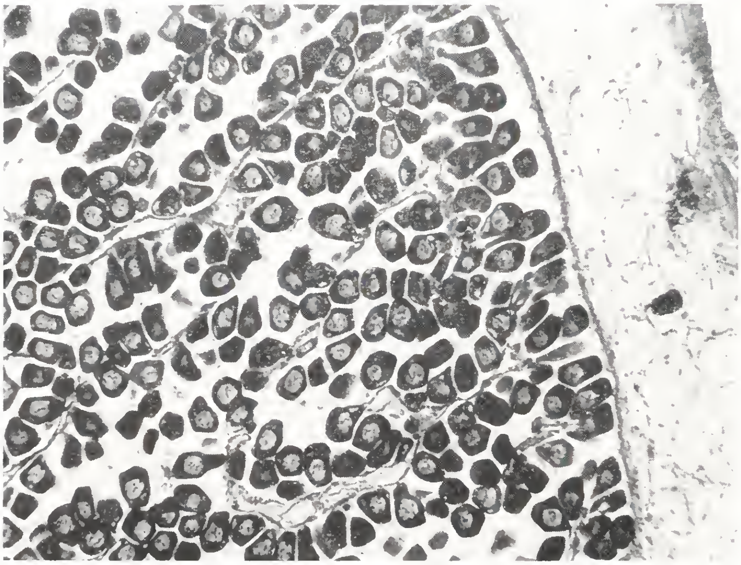


FIGURE 1. Section of gonad of Long Island Sound oyster preserved late in September, showing complete recovery from spawning. Small, undeveloped follicles, typical for post-spawning condition of oysters, are surrounded by masses of cells of the connective tissue. × 110.

FIGURE 2. Section of gonad of Virginia oyster preserved late in September, still containing fully developed undischarged eggs. × 110.

present in very large numbers, appeared to be abnormal. The color of the gonads changed from creamy white to grayish yellow, and instead of being soft and oozing, as was often noticed during the examination of September 27, the gonad material was hard. In almost all instances resorption of gonad material was in progress, the number of leucocytes and phagocytic cells being extremely large.

The other half of the New Jersey oysters had their gonads completely or almost completely devoid of spawn. Whether these oysters discharged the spawn in a normal way or whether their gonads were resorbed after failure to spawn, remained undetermined.

Somewhat the same conditions were observed in the two Virginia groups. However, only one out of 20 oysters had completely empty gonads. The others had either entirely undischarged gonads, or gonads that still contained large quantities of spawn. It was quite clear that at least 8 oysters of the 20 had never spawned (Table I). The thickness of the gonadal layer of the unspawned oysters was 3.0 mm. or more.

Our controlled experiments in Milford Harbor were collaborated by observations made on the oyster beds of Long Island Sound. A schooner load of oysters native to the Seaside of Virginia, south of Chincoteague, was brought to Connecticut by F. Mansfield and Sons Oyster Company and planted on the cultivated beds of the Company in Long Island Sound, south of West Haven, at a depth of approximately 10 feet. Examination of the gonads of these oysters made on several occasions late in the fall showed that many oysters did not release their spawn, while the local oysters living on the same bed were completely spawned by the middle or the end of September.

In general, our experiments have shown that the oysters which originated in Massachusetts or Long Island Sound, *i.e.*, in the more northern latitudes of the range in which *C. virginica* is found, spawned in Milford Harbor, Conn. during the summer of 1950, discharging all the spawn. However, the majority of the oysters that originated in warmer regions, New Jersey or Virginia, either did not spawn at all or discharged only a portion of the accumulated spawn. This difference indicates that the breeding temperature requirements of the northern oysters are somewhat lower than those of the southern groups. It also suggests that some physiological requirements of oysters of different geographical districts may be decidedly different. Consequently, it may be logically assumed that these groups of oysters, although all belonging to the same genus and species, *Crassostrea virginica* Gmelin, may, nevertheless, be distinct physiological races.

If, as our observations seem to indicate, there are different physiological races among the population of *C. virginica* of our Atlantic Coast, which require different temperatures for spawning, then, in addition to the theoretical interest of these observations, we are immediately faced with a number of considerations which may arise in connection with transplantation of oysters from one geographical district to another, especially a far removed one. For example, it has been shown that the oysters of Long Island Sound may begin to spawn at a temperature as low as about 16.0° C. (Loosanoff, 1939). Were these oysters transplanted to southern areas, they, as pointed out by Stauber (1950), would, under the new temperature conditions, begin to spawn far ahead of the local population. It is true that since spawning of a single oyster may continue for several weeks, some of the northern oysters may, at the end of their spawning period, crossbreed with the southern ones, which, requiring a higher temperature, would begin spawning later in the season. Nevertheless, because of the absence in the water of gametes of southern oysters during the largest part of the spawning of the northern races, most of the progeny of the northern oysters should remain to be of pure northern strain.

On the other hand, the majority of southern oysters transplanted to Long Island Sound, or other bodies of water in the north where the temperature seldom reaches and is practically never maintained for a prolonged period at 25.0° C., would not spawn. Such oysters, naturally, cannot be counted upon to contribute to the future increase of the local population.

Finally, the oysters with unspawned or partially resorbed gonads, of the type which were found in our samples of the southern groups on September 27 and October 25, 1950, would be virtually unsalable because of their poor quality. Obviously, the presence of southern oysters on the northern beds may not always be desirable because, while competing with northern oysters for space and food, they might neither participate in the increase of the local oyster population nor present a desirable food product.

In conclusion it should be mentioned that it is very fortunate for our studies that the summer of 1950 was comparatively cool, thus preventing the temperature of the water of Long Island Sound and particularly of Milford Harbor, from reaching an unusually high level, as happened during the record-breaking hot and long summer of 1949 when a temperature of 25.0° C. and higher was maintained for long periods. Under those conditions, of course, all oysters, including those of the three southern areas, would have spawned completely, leaving no evidence of the different temperature requirements of the different groups. In the past we found such to be the case with one group of oysters received from Eastern Bay, Maryland, which was kept in very shallow water in Milford Harbor near the shore where the temperature on some occasions was high enough to permit them to spawn normally.

We wish to express our thanks to our colleague, H. C. Davis, for his participation in the examination of gonads of the oysters, and to our friends in different parts of the country who were kind enough to ship us oysters for these studies.

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THE EFFECT OF PARROT FISH THYROID GLAND ON METAMORPHOSIS IN FROG TADPOLES

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The functions of the thyroid are not as well known in cold-blooded vertebrates as in birds and mammals. Thyroid experiments produce less striking results in lower vertebrates than in warm-blooded animals, with the exception of those concerned with metamorphic changes in amphibians. Perhaps because of this the number of experiments designed to determine what the gland does in fishes, amphibians and reptiles is relatively few. A better knowledge of what the thyroid does in these forms is desirable not only because such knowledge would enable us to interpret more completely the activities of these animals, but also because it might throw considerable light on any non-metabolic role that may be played by the thyroid in birds and mammals. As far as fishes are concerned, the diffuse nature of the thyroid gland in most bony fishes makes it difficult to study. Its scattered follicles can neither be removed to produce hypothyroid states nor can extracts of it be prepared that are free from contamination with other tissues. A large encapsulated gland has been described in the swordfish (Addison and Richter, 1932) but this material is difficult to obtain and no studies have been made with it. Several of the parrot fishes so common in subtropical and tropical waters also possess a large, encapsulated thyroid which can be removed free from surrounding tissues (Matthews, 1948), and these fish are fairly easy to obtain. A number of experiments have already been carried out on this material (Matthews and Smith, 1948; Smith and Matthews, 1948). The experiments presented here were designed to determine whether or not the parrot fish thyroid can accelerate metamorphosis in tadpoles, and, if so, whether this effect can be reduced or abolished by treating the fish with an antithyroid drug before removing the gland.

MATERIAL AND METHODS

Second year tadpoles of *Rana clamitans* collected in and near Williamstown were used as test animals. One experiment was run on animals collected in April, but in all other cases the tadpoles were collected in September and October. Individuals with hind limb buds of one to three mm. were selected and placed in small aquaria with ten animals and two and one-half to three liters of water in each.

Several of the external changes that occur as metamorphosis proceeds have been used to test thyroid preparations or other iodine-containing compounds. Shortening of the gut (Swingle, 1918), appearance of limb buds (Romeis, 1923), and decrease in volume (Gaddum, 1927) have all been used, as well as growth of hind limbs and decrease in total length. Some of these changes appear to be more accurate than others for quantitative tests, particularly the shortening of the gut. On the other hand reduction in total length and growth of the hind legs are easier to measure and can be made as often as desired on the same individual, and these external changes were chosen as indicators of the rate at which metamorphosis was progressing in the present experiments. Measurements were made with calipers every seventh day, the animals lying on wet filter paper, and the progress of metamorphosis was then recorded by noting any increase in the hind limb total length ratio.

Determination of the threshold level necessary to initiate metamorphosis was not made. With feeding methods it is difficult to estimate with any accuracy the amount eaten, and practically impossible to determine how much absorption of dissolved material occurs through skin and gills. For such quantitative determinations injection methods are preferable. These were abandoned in the present experiments after injection of thyroid preparations dissolved in basic solution had resulted in high mortality.

Thyroids of the rainbow parrot fish (*Pseudoscarus guacamaia*) were collected at the Bermuda Biological Station and dried either immediately in an oven at 50° C. or after preservation in acetone. Several glands from untreated animals were collected, and also one from a fish that had received ten daily injections of 20 cc. of a solution of propylthiouracil saturated at pH 8.0 before removal of the gland. After drying, the glands were pulverized, mixed with flour in the proportions of 300 mg. of thyroid powder to 1 gm. of flour, and enough water was added to form a thin paste which was then dried in thin sheets. This material was fed in excess by crumbling it into the tanks. Filamentous green algae or Anacharis were also added. At forty-eight hour intervals the aquaria were cleaned and fresh water and food were added. Control animals were given food prepared in the same way by mixing dried tail muscle or dried auricle with flour to form a paste which was allowed to dry as before.

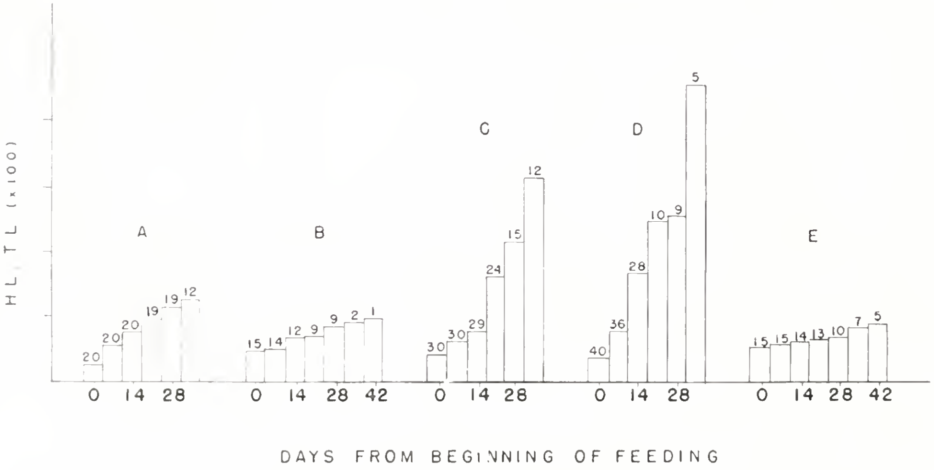

FIGURE 1. Rate of metamorphosis, as expressed by hind limb/total length ratios, of second year tadpoles of *R. clamitans* when fed preparations containing: A, Oven-dried fish muscle; B, Oven-dried parrot fish auricle; C, Oven-dried parrot fish thyroid; D, Acetone-dried parrot fish thyroid; and E, Oven-dried thyroid from a propylthiouracil-treated parrot fish. The figures at the top of the columns refer to the number of animals contributing to each average.

RESULTS

The effects of feeding these substances were studied on 130 tadpoles. Forty were fed oven-dried thyroid, 40 acetone-dried thyroid, and 15 were given thyroid from a propylthiouracil-treated animal. Of 35 control animals, 20 were fed tail muscle preparations; the other 15 were given food prepared from auricle tissue. The results on spring and fall animals showed some differences, but these could be interpreted as due to the small number of animals included in each experiment rather than to differences in threshold in the spring and fall tadpoles. At any rate the differences were not marked enough to justify separate presentation; consequently, the

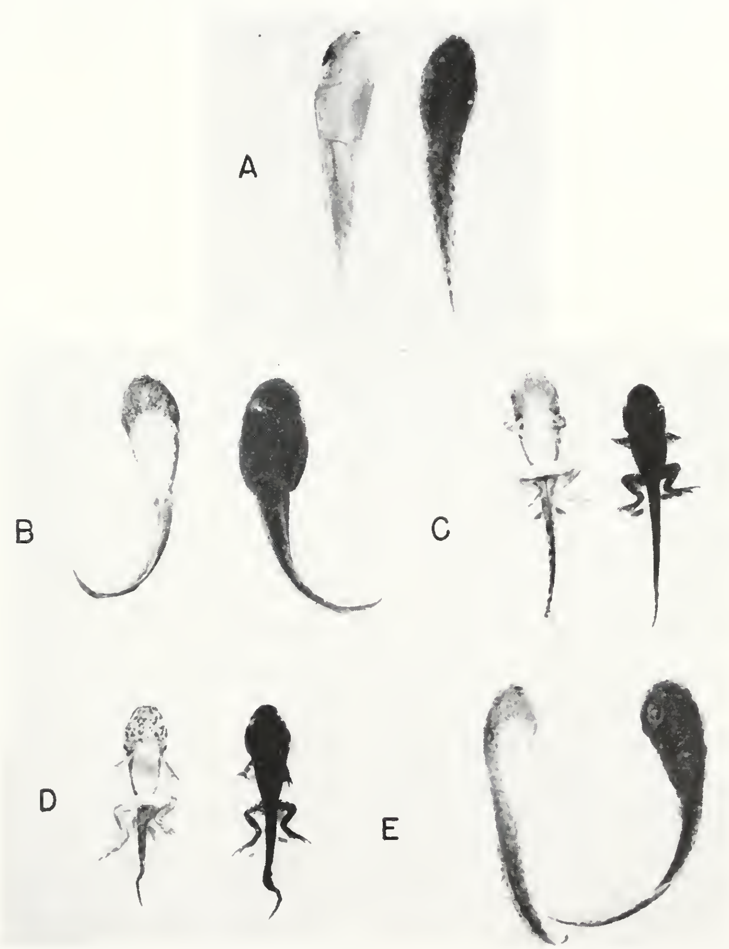

FIGURE 2. Second year tadpoles of *R. clamitans* after 28 days of feeding with preparations containing: A, Oven-dried fish muscle; B, Oven-dried parrot fish auricle; C, Oven-dried parrot fish thyroid; D, Acetone-dried parrot fish thyroid; E, Oven-dried thyroid from a propylthiouracil-treated parrot fish.

results of both spring and fall experiments are summarized and presented together in Figure 1. The height of each column represents the average ratio (hind limb/total length) of all animals in a given experiment on a particular day after feeding began. The number of animals contributing to each average is listed above each column. Since some animals died during the course of the experiments, the figures for the later parts of each run represent fewer cases.

Observable differences in the shape of the body of the tadpole occur late in the first week, the animal becoming more triangular in appearance. A change in the hind limb/total length ratio, as compared with that of the controls, also occurs at the end of the first week in the case of the acetone-dried preparations, but not until the third week with oven-dried thyroid. The differences between control and thyroid-fed animals became increasingly more marked in both series as feeding progressed. The effect of the acetone-dried preparations not only appeared earlier but was more marked at each period when measurements were taken. In contrast to these results the tadpoles fed thyroid from a propylthiouracil-treated fish behaved like the controls. The differences in external appearance in the various groups at the end of 28 days are shown in Figure 2.

DISCUSSION

It is clear from these experiments that the parrot fish thyroid accelerates metamorphosis in amphibians. A number of workers have suggested that the fish thyroid may be involved in the marked bodily changes that many fishes undergo during growth and sexual maturation. In the eel, for example, both Sklower (1930) and von Hagen (1936), after studying morphological changes in the thyroid, concluded that it was concerned with, if not responsible for, the pronounced changes that occur as the eel progresses from larval to glass eel stage. Sklower also suggests that the flounder thyroid controls metamorphosis in this animal. Some recent experimental work supports this view. Landgrebe (1941) found that the injection of ox thyroid into salmon and trout produced more rapid smoltification, a result that has been confirmed for salmon by La Roche (1949) and for rainbow trout by Robertson (1949). La Roche in fact suggests that it is the extra iodine present in sea water which enables the thyroid of the salmon parr to release the hormone that is responsible for smoltification. While the present experiments were carried out on amphibians rather than fishes, they do at least demonstrate that the thyroid of the parrot fish is capable of accelerating metamorphosis in tadpoles. Similar but less marked results have been obtained twice before with teleost material. Sembrat (1927) implanted thyroids of Cyprinus into tadpoles of *R. temporaria* and found that metamorphic changes began in 5 to 10 days as compared with 8 to 23 days in controls implanted with muscle and other tissues. Gunthorp (1932) also tested this effect by feeding flounder thyroid to tadpoles of *Hyla regilla*. His animals metamorphosed earlier than those fed beef preparations, but the effect here, too, was slight. Neither of these reports describes how the thyroid was removed nor do they indicate how much gill and other tissues may have been removed with it. Contamination with inactive tissues may have diluted the concentration of active material in these cases and might explain the smaller effect produced by them than by the parrot fish thyroid.

Either iodine or potassium iodide will accelerate metamorphosis of amphibian larvae, but diiodotyrosine is much more effective than either, and thyroxine produces the most striking results of all iodine-containing compounds (Allen, 1938). No accurate figures are at present available for either the organically bound or total iodine content of fish thyroids. The correlation of their accelerating effect on metamorphosis with their iodine content must await chemical analyses of this material.

It has been clearly established for the higher vertebrates that propylthiouracil and other antithyroid drugs of this type prevent the synthesis of thyroxine, apparently by blocking the oxidation of iodide to iodine. Hence the thyroids of animals treated with these compounds contain only small amounts of iodine, most of which is inorganic. The results of the present experiments indicate that such drugs have a similar action in fishes, since the thyroids of propylthiouracil-treated fishes do not accelerate metamorphosis.

Summary

The parrot fish thyroid markedly accelerates metamorphosis when fed to tadpoles of *Rana clamitans*. The thyroid of a parrot fish which had been treated daily for several days with propylthiouracil had no effect on metamorphosis.

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A NEW COMMENSAL HYDROID OF THE MANTLE CAVITY OF AN OYSTER[1]

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During the course of studies on the biology of the "mangrove oyster," *Crassostrea rhizophorae* (Guilding), in Puerto Rico, a number of oysters were found with the mantle cavity infested by an undescribed commensal hydroid (Mattox, 1949). The unusual habitat of this hydroid and its correlated adaptive features are not entirely unique, for two similar hydroids have been found in Italy, both also living in the mantle cavity of edible pelecypods. The hydroid here considered is recognized as belonging to the same genus as those found in Italy and is designated as *Eugymnanthea ostrearum.*

The Genus Eugymnanthea

Palombi (1936) established the genus *Eugymnanthea* in naming a new species of hydroid found within the mantle cavity of the lamellibranch, *Tapes decussatus,* in Lago Fusaro, near Naples. This hydroid, *Eugymnanthea inquilina* Palombi is the generotype by monotypy. Cerruti (1941) described a similar hydroid from *Mytilus galloprovincialis* at Taranto. Unaware of Palombi's work he named this *Mytilhydra polimantii,* but upon learning of *E. inquilina* altered the name of his species to *Eugymnanthea polimantii.* Our species is similar to the other two and must be placed in the same genus.

The following are the features common to the three species of *Eugymnanthea* and constitute a definition of the genus. Athecate, solitary polyps, lacking hydrorhizae and gonothecae, possessing a basal disc, a single whorl of tentacles, and producing from near the base, either sporosacs, or medusae of the leptomedusan family Eucopiidae. The three known species are commensal within the mantle cavity of pelecypods.

Eugymnanthea ostrearum nov. sp.

Description of the hydroid (Fig. 1 A). The polyps are simple, solitary, tapering toward the base, having a maximum length of 3.8 mm. (the average of 20 specimens, taken at random, was 1.54 mm.), having a maximum diameter of the column of 0.35 mm. There are as many as 35 filiform tentacles (average 30) in a single whorl. There is a slight intertentacular membrane which, on the largest polyp, was 0.18 mm. in width. The hypostome is a slightly elevated cone which does not extend beyond the limits of the intertentacular membrane. A striking feature is the basal or pedal disc, a flattened, slightly concave expansion of the base of the column.

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[1] Contribution from the Department of Biology, College of Agriculture and Mechanic Arts, Mayaguez, Puerto Rico (Mattox), and Contribution No. 474 from the Department of Zoology, Indiana University, Bloomington, Indiana (Crowell).

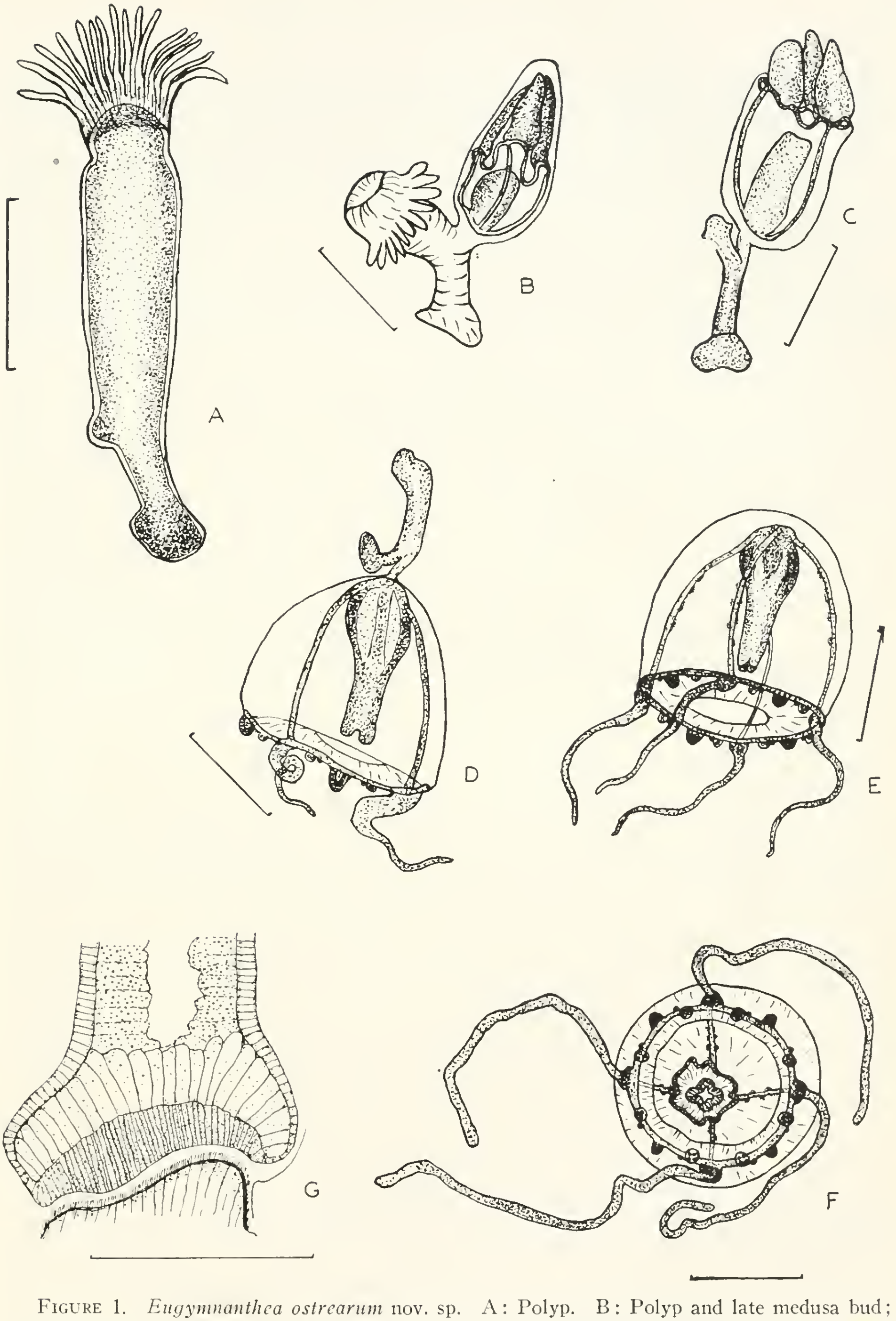

FIGURE 1. *Eugymnanthea ostrearum* nov. sp. A: Polyp. B: Polyp and late medusa bud; the oral region of the polyp shows an unusual eversion. C: Medusa bud just before it began to swim and at a stage when the polyp had become reduced. D: Medusa two hours after it became free swimming, with the polyp still attached. E: Medusa one day after liberation. F: Medusa in oral view five days after liberation. G: The basal disc on the ciliated epithelium of the oyster; the scale line is 0.1 mm. for G. For A to F the scale lines are 0.5 mm.

There are no hydrorhizae, nor is there evidence of any periderm (perisarc). Nematocysts are present over the whole column and in batteries on the tentacles. They are small atrichous isorhizas, with capsules averaging 5 micra in length, and threads not much longer than the capsules. Buds near the base produce medusae.

Polyps are found adhering to mantle, foot, gills and labial palps of the "mangrove oyster," *Crassostrea rhizophorae* (Guilding), on the southern side of the Laguna Rincon at Boqueron, Puerto Rico.

Description of the young medusa (Figs. 1D, 1E, 1F). Medusae were kept in the laboratory for eight days. During this time no features appeared not observable soon after liberation. So far no medusae have been found in plankton samples from the Boqueron lagoon.

The young medusa is ovoid, slightly higher than wide, with a maximum height of 3.0 mm. There are four simple radial canals and a circular canal. Four simple perradial tentacles are borne on conspicuous hollow bases. At the time of liberation there are four interradial tentacle bulbs, and by the fifth day 8 very small adradial bulbs appear. Perhaps older specimens develop additional tentacles, but this has not been observed. There are 8 adradial statocysts, each with 2 to 6 statoliths. (These are perradial to the adradial bulbs when the latter appear.) There are no ocelli nor cirri. The manubrium is of moderate size extending about to the opening of the well developed velum. The mouth is surrounded by four simple lips. At the time of liberation there are no gonads; but specimens five days old show indications of gonad development on the oral surface of the radial canals. The nematocysts are similar to those of the hydroid. Specimens were obtained from the hydroid.

Cotypes are in U. S. National Museum: the medusae No. 49808, the polyps No. 49809.

In Table I we have compared the medusae of the three species of *Eugymnanthea* as well as *Eucope parvigastra,* a medusa described by Mayer (1910) but one whose hydroid is unknown. Although such a comparison points up the obvious differences among these medusae, a consideration of these differences suggests that they are really quantitative and directly correlated with the time at which gonads develop. If gonads appear early the medusa remains small and tentacles and manubrium do not develop. On the contrary, if gonads develop late, the feeding parts of the medusa (tentacles and manubrium) are developed. Similar gonophore series are exemplified by other hydroids. The neatness with which *Eucope parvigastra* fits this series suggests that its unknown hydroid might be found in a bivalve of the Tortugas region. However, Dr. W. J. Rees (personal communication) has expressed doubt that there is any connection between *Eucope parvigastra* and *Eugymnanthea.*

TAXONOMIC CONSIDERATIONS

Although *Eugymnanthea* lacks periderm and most other features of typical Calyptoblastea, it produces a leptomedusa, and so must be regarded as a calyptoblastic hydroid. There are a few other hydroids which are athecate but produce leptomedusae. Some of these medusae belong to the subfamily Eutiminae, family Eucopiidae, and Stechow (1924) has proposed to place such hydroids in the subfamily Campanopsinae, of the family Heleciidae. This will not serve for *Eugymnanthea ostrearum* since its medusa belongs in the subfamily Obelinae of Eucopiidae.

Possibly (as Palombi, 1936, suggests) *Eugymnanthea* should be placed in a new subfamily of Haleciidae, having affinities with Stechow's Campanopsinae. It is impossible to include the hydroid in any of the families of hydroids as defined by Fraser in "North American Hydroids" (1937, 1944). Since the absence of periderm is probably an adaptive character related to the commensal mode of living, little significance should be placed upon it in attempting to determine natural relationships.

Fortunately the medusa is readily classified. It fits the genus *Eucope* (Mayer, 1910) in all anatomical features. However, we regard it as generically different for the following reasons. Its polyp is so similar to that of *Eugymnanthea inquilina* and *E. polimantii* that it must be placed in the same genus. Their medusae do not fit the definition of *Eucope* but are similar to *Agastra*. Furthermore none of the known hydroid stages of either *Agastra* or *Eucope* resemble the hydroid *Eugymnanthea*. Therefore we believe that the name *Eugymnanthea* should be applied to those medusae of the family Eucopiidae, subfamily Obelinae, which show resemblences to *Eucope* or *Agastra* and which are derived from polyps of the genus *Eugymnanthea* as previously defined.

Table I

Comparison of Medusae

Eugymnanthea inquilina Palombi	*Eugymnanthea polimantii* Cerruti	*Eucope parvigastra*	*Eugymnanthea ostrearum* nov. sp.
Naples, Italy	Taranto, Italy	Tortugas, Florida	Boqueron, Puerto Rico
Polyp in mantle cavity of *Tapes*	In mantle cavity of *Mytilus*	Polyp unknown	In mantle cavity of *Crassostrea*
Medusa 0.55 mm.	1.2 mm.	1.0 mm.	3.0 mm.
Spheroconical	Same	"Half-egg-shaped"	Same
8 adradial otocysts	Same	Same	Same
Not reported	3 otoliths, sometimes 1 or 2, rarely 4	Single spherical	2 to 6 otoliths
4 simple radial canals	Same	Same	Same
4 perradial tentacle bulbs	4 perradial bulbs 4 small interradial bulbs	4 perradial bulbs	4 well developed perradial tentacles on large bases, 4 interradial bulbs, 8 small adradial bulbs
Gonads on radial canals. Large oval, shedding gametes before or soon after liberation	Same position. Same size as *E. inquilina*, hence relatively smaller, shedding gametes after liberation	Same position. Small	Same position. Not developed at time of liberation
Manubrium lacking	Manubrium lacking	"Very small, a mere tube cruciform in cross section"	Manubrium simple, extending to velar opening
Mouth lacking	Mouth lacking	Mouth present	Mouth present
Lips lacking	Lips lacking	4 simple lips	4 simple lips

Reproduction of the Hydroid Generation

The method by which polyps produce other polyps has not been studied carefully in any of the species of *Eugymnanthea*. During the spring and early summer

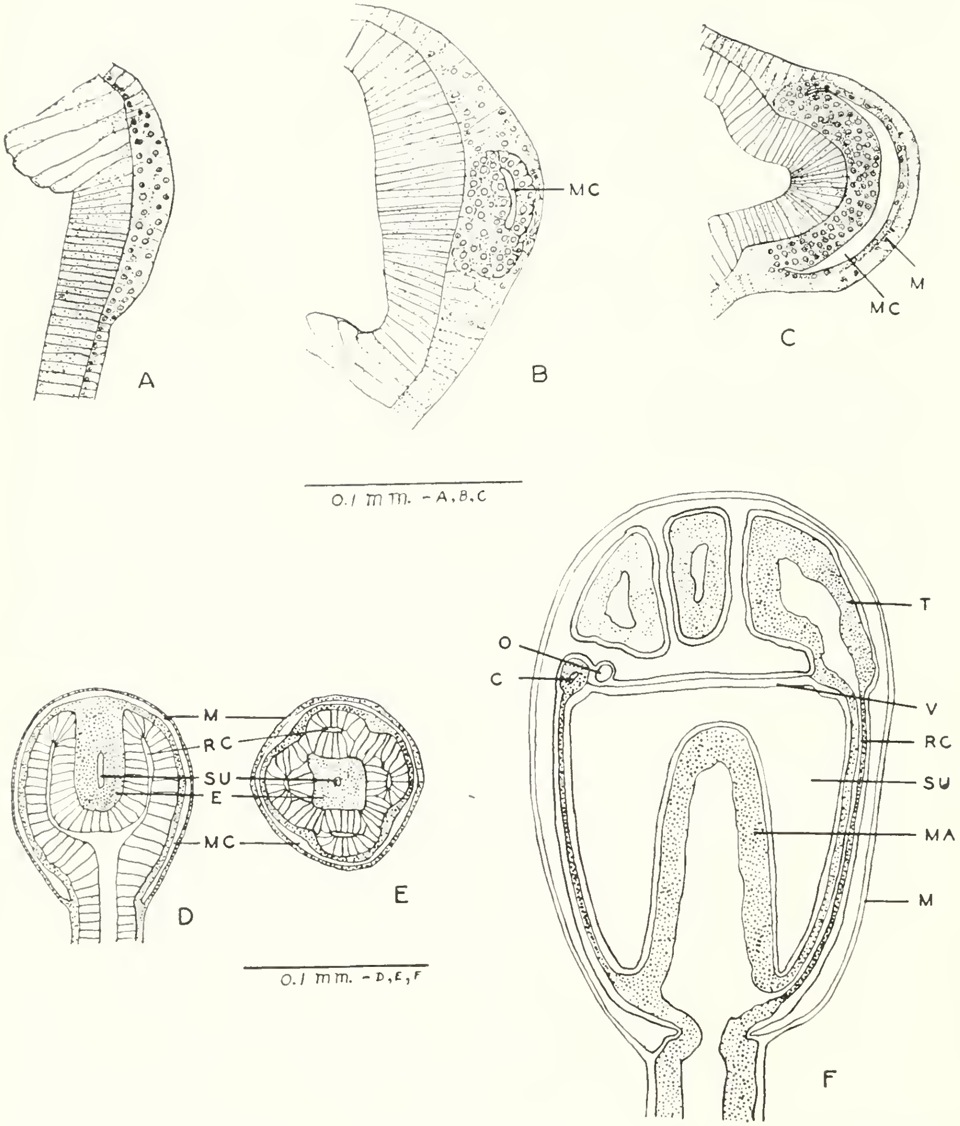

FIGURE 2. Development of the medusa. A: Early epidermal placode. B: Establishment of mantle cavity within the epidermal placode. C: Evagination of the bud and extension of the mantle. D, E: Diagrammatic radial and transverse sections of a stage at which the prospective sub-umbrella cavity is forming within the well established entocodon; epidermis stippled. F: Medusa just prior to liberation; on the left adradial section to show endodermal lamella and statocyst, on the right perradial to show radial canal and tentacle base; endodermis stippled. c, circular canal; e, entocodon; m, mantle; ma, manubrium; mc, mantle cavity; o, otocyst; rc, radial canal; su, prospective or actual sub-umbrella cavity; v, velum.

Cerruti (1941) found groups of polyps, usually two or three, once as many as 7, attached to one another and arising from one basal disc. He says that these arise by budding and that later the polyps separate from one another. Palombi (1936) does not mention this type of reproduction but does show figures of two and of three polyps arising from a common basal disc. In some of our specimens a small polyp is joined to a larger one near the base of the latter. In others two polyps of nearly equal size share a common basal disc. Lacking direct continuous observations we cannot be positive, but all indications suggest the following method of reproduction. The second polyp arises as a bud near the base of the first, just as in related campanularians. The bud becomes a polyp which grows. Finally the two polyps split the basal portion of the column and the basal disc so that when separation occurs each obtains part of the basal disc. Such a combination of budding followed by fission does not occur in any other hydroid, as far as we know, but it is appropriate for a species in which the basal disc is so important.

Development of eggs has not been observed for any species of *Eugymnanthea*. However, Cerruti (1941) observed, in October, on the mantles of some of the mussels some little, somewhat spherical bodies and some very small polyps. He interprets the small bodies as due to the attachment of larvae which would soon transform into polyps.

Development of the Medusa

Medusa buds arise singly near the basal end of the column, a polyp bearing only one medusa at a time. The changes of form and structure during development are similar to those described for other medusae having a comparable structure at the time of liberation. Since the other species of *Eugymnanthea,* unlike *E. ostrearum,* have gonads but no manubrium nor tentacles when liberated, it follows that our species is more like *Clytia,* for example, than it is like the other two species of *Eugymnanthea,* in respect to its developmental stages. (For *Clytia* see Goette, 1907.)

In the earliest stages of bud formation both epidermis and gastrodermis become modified. The epidermis becomes thickened as a placode (Fig. 2A). Its cells become less vacuolated and several layers deep. The gastrodermal cells beneath this placode have about the same height as those which lie between it and the basal disc, but are narrower. They have lost the vacuoles of typical gastrodermal cells but retain the conspicuous granules. By the time the placode has thickened so that it is about five cells thick, its cells have lost the vacuoles, and the underlying gastrodermal cells no longer show the large granules (Fig. 2B). The small size of the cells, their loss of vacuoles and granules, the increase in size of the placode and its evagination as a young bud all reflect high mitotic activity. This has been clearly shown for other developing medusa buds by Berrill (1949a, 1949b).

The mantle and mantle cavity are established very early through the rearrangement of epidermal cells of the placode. The mantle (sheath, ectotheca) is an epidermal covering of the developing medusa characteristic of most calyptoblasts. It is described as arising by delamination of irregular thickenings of the epidermis (Goette, 1907). In *Eugymnanthea* this delamination has more orderliness and precision than has been reported in other cases. Figure 2B shows an early mantle cavity. The cells which line the cavity appear to be oriented with reference to it, and its extension the result of their rapid multiplication. The mantle itself is two cell layers thick. The outer cells soon become vacuolated and continuous with the epidermal cells beyond the placode; while the inner cells resemble those of the rest of the placode with which they are continuous around the circumference of the mantle cavity (Fig. 2C). The mantle becomes thinner as the medusa develops and is ruptured at the time of liberation.

The epidermal cells of the placode soon alter their arrangement to produce the entocodon within which the prospective sub-umbrella cavity is formed. Figures 2D and 2E illustrate this stage. The entocodon is quadrilateral in cross section. It is flanked on its slightly concave sides by the endodermal sacs destined to form the radial canals, and is bordered at its corners by a single layer of endodermal cells which are continuous with the endodermal sacs. Subsequently the sub-umbrella space expands; the manubrium arises and extends into it. By these processes and the establishment of tentacle bases, velum and otocysts, the stage is reached, just prior to liberation of the medusa, illustrated by Figure 2F. The four tentacle bases are much larger than in the other species of *Eugymnanthea* and most other calyptoblasts. They have about the same proportions as those of *Clytia* (Goette, 1907).

During the late stages of the development of the medusa there is a striking reduction in the size of the polyp. It loses its tentacles by reduction and resorption, and diminishes in size to less than one fourth of the original size. When the medusa ruptures its mantle, begins to pulsate, and becomes free swimming it carries the small remnant of the polyp with it. This remnant drops from the medusa soon after the medusa begins its free life. The reduction of the polyp is illustrated in Figures 1C, 1D, and 1E. Palombi (1936) found a similar reduction of the polyp during the formation of the medusoids of *E. inquilina*, but Cerruti (1941) found no such reduction in *E. polimantii*. An analagous reduction occurs in *Obelia* and in *Campanularia* (Berrill, 1949b; Crowell and Rusk, 1950), where blastostyle production often causes regression of the nearby hydranths. A similar regression of the polyp of *Craspedacusta* accompanies the production of a medusa (Payne, 1924).

In most colonial calyptoblastic hydroids the production of a gonangium requires the cooperative support of many feeding hydranths. A single small polyp, like that of *Eugymnanthea*, is probably unable to furnish sufficient substance for the construction of a gonangium. As an alternative it produces a single medusa with a morphogenesis like that of one of the medusae arising on a blastostyle.

The Commensal Relationship

Oysters containing *Eugymnanthea* have been found at all seasons in the Boqueron lagoon. In 11 random samples, totalling 138 oysters, and extending from January to December of 1950, 12 per cent contained polyps. In heavily infested oysters the polyps are attached chiefly to the inner surface of the dorsal region of the mantle, but are also found on the foot, gills and labial palps. Some are scattered and isolated, others are found in large groups. Some oysters contain only a few polyps, others many. In one oyster 4.5 cm. long, 210 polyps were counted.

The most striking feature of *Eugymnanthea* is the conspicuous basal disc by which the polyps are enabled to remain attached to the host tissue. In detached specimens the disk appears circular and concave. In prepared sections, where it is attached, it shows both a tendency to be concave and at the same time to modify its shape to conform with folds of the host epithelium. The epidermal cells of the disc are high, narrow, heavily stained (hematoxylin), and non-vacuolated (Fig. 1G). The gastrodermal cells of the disc are large and lack the conspicuous granules of unmodified gastrodermis. Although the disc resembles the pedal disc of hydra superficially, it seems clear that the ability to adhere is not due to secretion of a mucous material which could hardly be effective on the slimy epithelium, but rather to suction achieved through the muscular activity of one or both cell layers. In living oysters the polyps sometimes become detached, then re-attach at other sites.

Specimens are attached both on naked and on ciliated epidermis of the host. The cilia do not appear to be damaged. There is no evidence of penetration into host tissue nor alteration of it. Cerruti (1941), in *E. polimantii*, reports loss of cilia of the mantle where the discs attach, and the presence of granules in the cytoplasm of the epithelial cells of the host mussel. The disparity between our observations and his may be due to a real difference between the hydroid-host relationship in the two pairs of species, or it may be that such damage may sometimes occur, sometimes not, in both species.

The mantle cavity of a pelecypod is obviously an excellent dwelling place for a small hydroid. It provides protection and an abundant current of water. The only disadvantage would seem to be the difficulty in remaining there. Most hydroids could not do so; *Eugymnanthea* has the necessary adaptation, the basal disc. Periderm (perisarc) is unnecessary in such a small and well protected polyp. Stolons (hydrorhizae) would be useless. The modifications in the methods of producing additional polyps and in producing medusae, discussed earlier, are also appropriate to the peculiar habitat of this hydroid.

We wish to thank Dr. W. J. Rees of the British Museum for helpful suggestions, particularly in reference to the taxonomy.

SUMMARY

1. *Eugymnanthea ostrearum* nov. sp. is a small, solitary hydroid living, attached by a basal disc, within the mantle cavity of the oyster *Crassostrea rhizophorae* (Guilding), in Puerto Rico. Two similar species have been described from Italy.

2. Its taxonomic position as a hydroid is somewhat uncertain since it lacks nearly all of the characters used in hydroid classification. It produces a leptomedusa similar to *Eucope*, and as a medusa belongs to the family Eucopiidae, subfamily Obelinae.

3. It shows the following features which may be regarded as adaptations appropriate to its commensal existence within the mantle cavity of the oyster: basal disc, absence of perisarc and stolons. Since the polyps are solitary there can be no gonangia.

4. Polyps are produced by budding followed by a fission of the basal disc. The development of the eggs has not been studied.

5. The development of the medusa is not unusual except in the way in which the mantle is established. It arises in a more sharply defined manner than that described in other forms by previous observers.

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THE SOLUBILITIES OF SOME VERTEBRATE FIBRINOGENS IN PLASMA–ETHANOL MIXTURES[1]

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Fibrinogen, the plasma protein which forms fibrin under the action of thrombin, is the least soluble major component of plasma. It is a euglobulin precipitating when the ionic strength is reduced much below the physiological level and is "salted out" of mammalian plasmas at such relatively low ionic strengths as half saturated (3.2 *M*) sodium chloride (Hammarsten, 1879; Florkin, 1930) and 10.6 per cent (0.75 *M*) sodium sulfate (Howe, 1923). It is also precipitated in dilute solutions of ethanol at low temperatures (Edsall, Ferry and Armstrong, 1944; Morrison, Edsall and Miller, 1948) and we have used this behavior as a criterion of solubility.

Materials and Methods

Fibrinogens from representatives of four vertebrate classes were studied. Blood was usually obtained by exposure of the heart and direct heart puncture, although some animals were bled by heart puncture through the chest and others from the canulated carotid artery. Siliconed needles and syringes containing a tenth volume of 10 per cent sodium citrate as an anticoagulant were used. Blood was chilled on ice usually within 10 minutes and stored in siliconed or plastic containers until centrifugation, which was carried out in two stages with the second at 5000 r.p.m. to insure removal of fine particulate material which would otherwise be carried down in the clot. This procedure usually produced a very clear straw or yellow plasma although in certain individual frogs and turtles a substantial amount of hemoglobin was present in the plasma even with these precautions. This hemolysis was not correlated with differences in technique, so it may represent differences in the physiological condition of the animals about whose background little was known.

Since as little as one cc. of blood was the most that could be obtained from certain of the smaller animals, it was necessary to adapt procedures to small volumes. One cc. of plasma was diluted with an equal volume at an appropriate concentration of ethanol (by volume) and sodium chloride to maintain the ionic strength constant at 0.15 and the mixture was equilibrated for two hours at 0° C. The precipitate and supernatant phases were then separated by refrigerated centrifugation in the same tube and decantation. The precipitate was dissolved in 2½ cc. of pH 6.4 phosphate buffer, ionic strength of 0.15, and two 1 cc. samples of this solution together with a 1 cc. sample from the supernatant were analysed. The fibrinogen was assayed gravimetrically after clotting with thrombin, syneresis and washing, drying and weighing

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[1] This is paper 6 of a series on "The formation of fibrin and the coagulation of blood" from the University of Wisconsin, supported in part by research grants from the National Institutes of Health, U. S. Public Health Service. This work was also supported by research grants from the Wisconsin Alumni Research Foundation.

(Morrison, 1947). Since the final clot weights usually ranged from 0.1 to 1 mg., a torsion balance sensitive to 0.005 mg. was employed. With these small amounts results usually agreed within 5 per cent. This represents a substantially greater error than had been obtained with 20 mg. clots (Morrison, 1947) of human fibrinogen. For mammalian fibrinogens a final concentration of 0.1 to 0.2 unit (Ferry and Morrison, 1947) per cc. of bovine thrombin [2] was employed. Bovine thrombin was also employed to clot fibrinogens of lower forms but since the heterologous reaction between fibrinogens and thrombins from different classes is much less effective (unpublished observations) larger amounts were necessary. A concentration sufficient to yield a 5 to 10 minute clotting time was usually employed and clotting was continued for two to four hours. Considerable fibrinolytic activity was sometimes encountered in plasmas from the lower forms and it was necessary to discard such series.

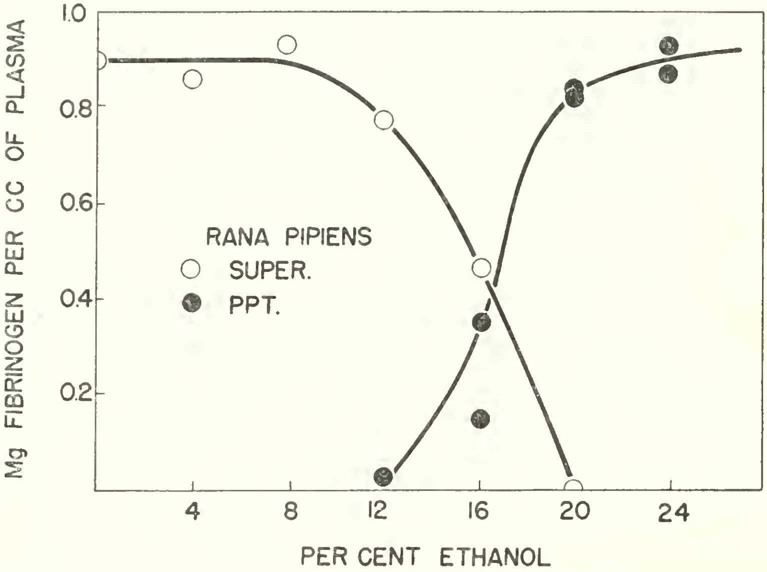

FIGURE 1. A representative series on 1 cc. samples of plasma showing the distribution of fibrinogen in the precipitate and supernatant phases with increasing amounts of ethanol. Open circles, supernatant phase; closed circles, precipitate phase.

Figure 1, showing the yield of fibrinogen in the supernatant and precipitate as a function of ethanol concentration, illustrates a typical experimental series on frog plasma using a total of 7 cc. of plasma. The sum of the supernatant and precipitate values usually was satisfactorily constant although at low fibrinogen levels low yields will be obtained (Morrison, 1947). For example in Figure 1 at 12 per cent ethanol this sum is only 90 per cent of the expected value. At high ethanol concentrations the yields tended to rise above the initial value presumably because of precipitation and/or denaturation of other plasma components with subsequent occlusion in the clot. In such cases the synerized clots were distinctly yellower.

[2] This material was supplied through the kindness of Dr. James Lesh of Armour and Company.

RESULTS AND DISCUSSION

Precipitation curves for three species of mammals, the rabbit, dog and cow, are shown in Figure 2. The points are sufficiently homogeneous for a single curve to represent the three species. Actually, successive series on individuals yielded as much variation as is shown between species in this figure. Human fibrinogen, which is 65 per cent precipitated at 8 per cent ethanol, fits quite well with these series.

Results on two turtles, the snapping turtle, *Chelydra serpentia* and the terrapin, *Pseudemys,* are shown in Figure 3. Reasonable correspondence is seen between individuals but there is a distinct difference between the two genera.

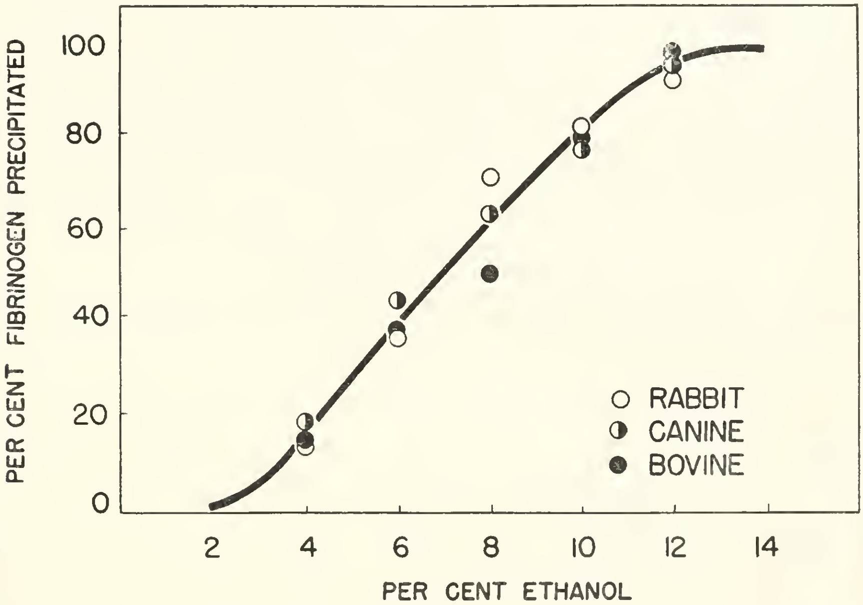

FIGURE 2. The precipitation of mammalian fibrinogens from their plasmas (per cent of the plasma level) as a function of ethanol concentration at 0° C. Open circles, rabbit; half circles, canine; closed circles, bovine. Each of the values for rabbit and dog represents an average of two series on different individuals.

Results on three amphibia, the bullfrog, *Rana catesbiana,* the green frog, *Rana pipiens,* and the mudpuppy, *Necturus,* are shown in Figure 4. Again, individuals of the same species fall together but distinct differences are seen between groups. The curve for the bullfrog suggests that two components with different properties were being precipitated, since 5 to 15 per cent of the material came down between 4 and 8 per cent ethanol while the bulk of the fibrinogen required considerably higher amounts of alcohol for precipitation.

Average curves for the species studied are assembled in Figure 5. The shapes of these curves are very similar although minor differences may be noted. It is immediately seen that the precipitation curves for the several classes stand in the same regular series which relates them phylogenetically, the mammalian fibrinogen

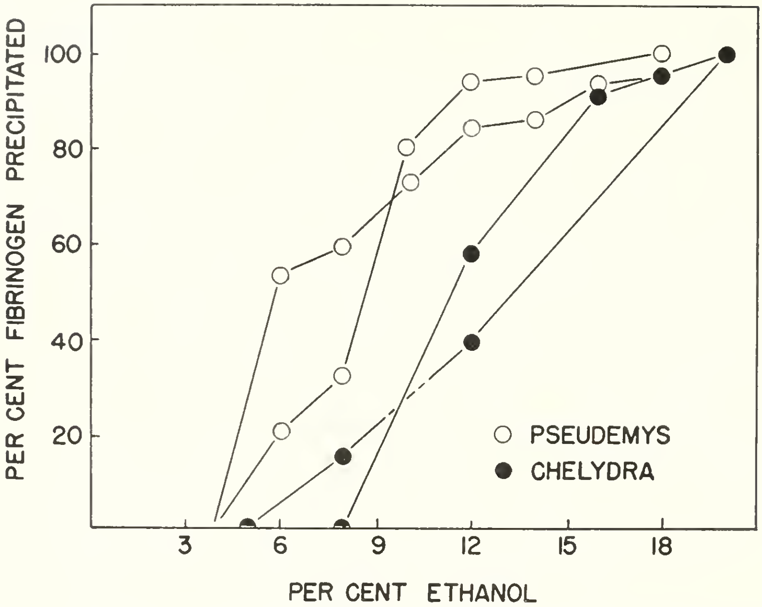

FIGURE 3. The precipitation of fibrinogen from two species of turtle plasma as a function of ethanol concentration at 0° C. Closed circles, snapping turtle, *Chelydra serpentina;* open circles, terrapin, *Pseudemys.*

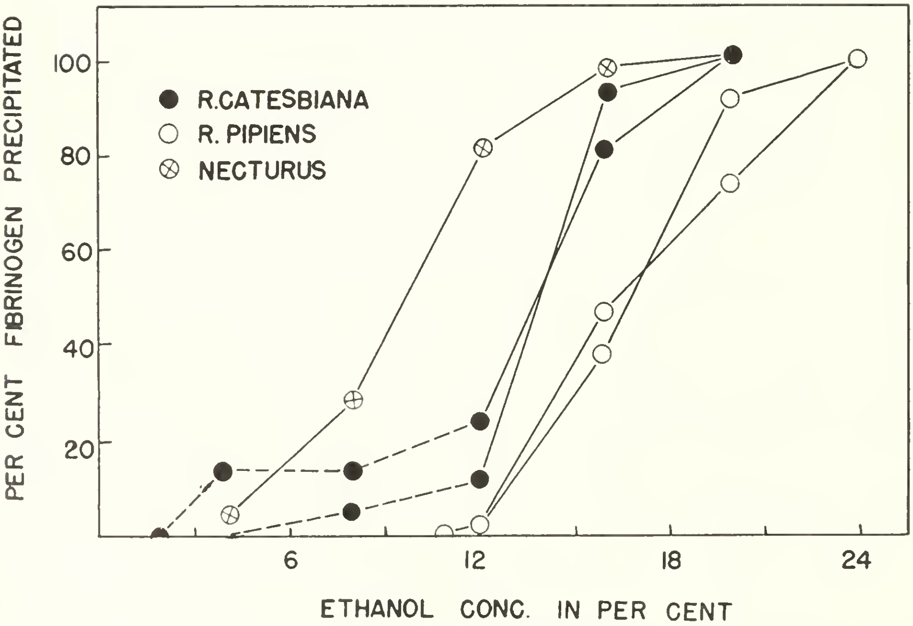

FIGURE 4. Precipitation of several amphibian fibrinogens from plasma as a function of ethanol concentration at 0° C. Open circles, grass frog, *Rana pipiens;* closed circles, bullfrog, *Rana catesbiana;* cross circles, mudpuppy, *Necturus.*

TABLE I

Average ethanol concentrations for precipitation of various fibrinogens from plasma

Group	Species	Series	% Ethanol for	
			50% ppt.	80% ppt.
Mammals	3	5	7	9
Turtles	2	4	11	13
Frogs	2	4	15	18
Carp	1	1	19	24

being least, and the fish fibrinogen (carp) most soluble. The ethanol concentrations required for 50 and 80 per cent precipitation, respectively, are summarized in Table I. Although the broad trend is evident, differences between species are of the same order of magnitude as are the differences in average values of the classes. And, indeed, the curve for the amphibian, *Necturus*, which is not indicated on Figure 5, falls more nearly with the turtles than with the frogs.

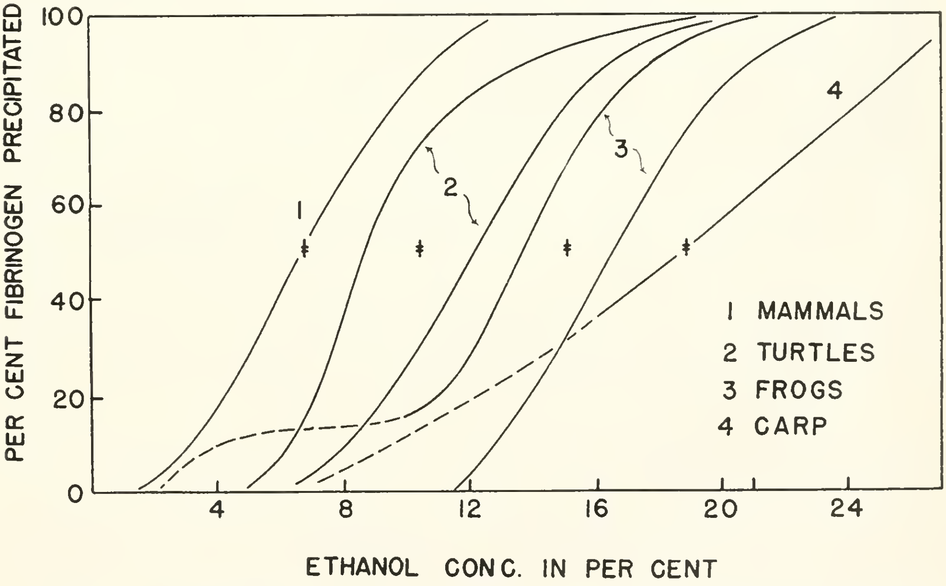

FIGURE 5. Average curves for the precipitation of fibrinogen as a function of ethanol concentration for the species shown in Figures 2, 3, and 4, together with a single curve for the carp. Symbols show the average values for 50 per cent precipitation for the four classes.

Although these precipitation curves reflect changes in solubility, they are not the same as solubility measurements since they will be influenced by the amount of the fibrinogen originally present in the plasma. However, since the curves are quite steep, variation in initial fibrinogen level will have a relatively small effect on the ethanol concentration required for 50 per cent precipitation. In Figure 6 the average results of measurements on the three classes have been presented in terms of actual solubility of fibrinogen in the supernatant phase at successive ethanol concentrations. The trend toward increasing solubility in the lower forms is again shown.

It is worth noting that this trend in fibrinogen solubility appeared to be followed by the other components since the mass of the fibrinogen-containing precipitate in the lower forms was not substantially larger than in the higher forms despite the considerably higher ethanol concentration. Plasma samples from two primitive fish, the garpike and the bowfin, which were unsatisfactory for fibrinogen assay, showed almost no precipitation even at 25 per cent ethanol.

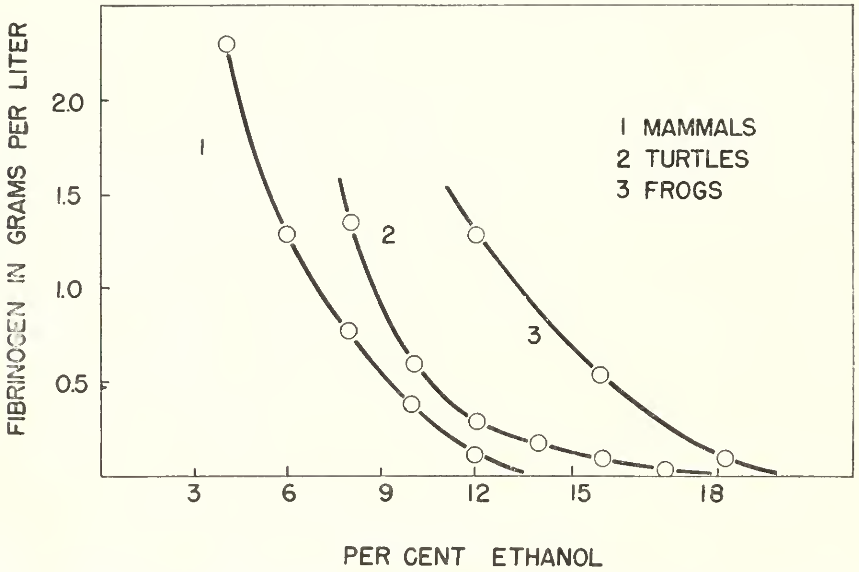

Figure 6. Average solubility curves for (1) mammal, (2) turtle, and (3) frog fibrinogens calculated from the supernatant values of the series in Figures 2, 3 and 4.

The significance of these differences in solubility in terms of molecular structure cannot be directly predicted. A more soluble molecule may also be a smaller molecule if the two are similar in other respects. Thus, in heterogeneous mixtures of high polymers the lower molecular weight fractions are the more soluble and, similarly, the products of protein cleavage are more soluble than the original protein. Besides their functional similarity, electrophoretic evidence suggests that the fibrinogens may be chemically similar. Deutsch and Goodloe (1945) reported a mobility of 2.6×10^{-5} cm.^{2} v^{-1} sec.$^{-1}$ for carp fibrinogen as compared to an average value of 2.43 (range: 2.1 to 3.2) for 12 species of mammals. Deutsch and McShan (1949) found no general shift toward components of higher mobility in studies on serum proteins from snakes, frogs and fish. However, their carp serum showed a shift toward components of higher mobility, perhaps correlated with the high albumin to globulin ratio reported for this species by Field, Elvehjem and Juday (1943). Such behavior, by reflecting a higher net charge on the molecule, might also account for the observed differences in solubility. Another piece of evidence which may suggest smaller molecules in the lower forms is the fact that the clots formed from these fibrinogens are invariably of the "fine" variety (Ferry and Morrison, 1947) showing little turbidity and synerizing with difficulty. These properties are considered to indicate small units of structure and small interstices such as might be expected from an aggregation of smaller molecules. Final conclusions must, however, await more careful characterization of these fibrinogen molecules by physical and other means.

Summary

The precipitation and solubility of fibrinogens from plasmas of four vertebrate classes by ethanol at 0° C. and 0.15 ionic strength was investigated. A regular series showing increasing solubility was observed as one went from mammals down through the lower classes.

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NITROGEN TENSION IN THE SWIMBLADDER OF MARINE FISHES IN RELATION TO THE DEPTH

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It has long been known that fishes with gas bladders occur down to very considerable depths. At 20 atmospheres depths they are still very common. Ling has been caught at 300 fathoms (60 atm.), cusk at 500 fathoms (100 atm.) (Bigelow and Welsh, 1924). Richard (1895) gives gas analysis data on fishes trawled for and possibly caught as deep as 1674 meter (170 atm.). Schloesing and Richard (1896) give analyses from fishes trawled at 900 and 1385 meters depths.

The gas pressure in the sea is only about one atmosphere at all depths. Since the free gases in the swimbladder are compressed according to the hydrostatic pressure, it is clear that we are dealing with gradients in the gas pressure between the outside water and the inside of the swimbladder of 20 to 50 to 100 and possibly more atmospheres. Obviously, therefore, at least one of the gases in the swimbladder must be secreted against very high pressures. Any general theory wishing to explain the gas secretion into the swimbladder must be able to account for this fact. We shall not enter into any discussion of the different theories advanced to explain the oxygen secretion, as they have been excellently reviewed in a paper by Koch (1934). It suffices here to point out that even the most generally accepted theory (Jacobs, 1930–34) requires an extrapolation of at least ten times beyond present knowledge in order to account for the known pressure performance of the gas gland.

It is well known that fishes at the surface generally have a gas composition in their swimbladder near that of atmospheric air (Jacobs, 1930; Hall, 1924; Black, 1942). It has also been known for a long time that, in general, the oxygen percentage increases as the depth gets greater. Biot (1807, quoted by Tower, 1901) reports 87 per cent oxygen in *Trygla lyra* from 500 fathoms, and high oxygen values in several other species from lesser depths. Tower (1901) verified these findings in other species. Richard (1895) and Schloesing and Richard (1896) found 75–85 per cent oxygen in the swimbladder of several deep sea fishes mentioned above. In other cases, however, a very low oxygen concentration was found in fishes caught at 7–8 atmospheres depths (Hüfner, 1892). It seems that no systematic study has so far been made of the relation of depth to the gas composition of the swimbladder. Such knowledge is necessary for the characterization of the performance of the gas gland. We have hence undertaken a study of the relation of depth to the partial pressure distribution of the gases of swimbladders from a variety of saltwater fishes.

Material

sic !

The present investigation was conducted from U. S. Bureau of Fisheries, Woods Hole, Mass., and from the Lerner Marine Laboratory, Bimini, Bahamas. The northern material was obtained on board MS Albatross III, Cruise No. 38, August 22–31, 1950, off the New England coast. The fishes were caught by bottom trawl which would drag the bottom for 30 minutes. It would take from 15–30 minutes from the time the trawl left the bottom until the gas sample was secured. Since the trawl was open on its way down and up, some fishes might have been caught in midwaters. The bottom depth was accurately known from a continuously recording fathometer. The shallow water fishes were caught by dragnet on shallow beach flats or by means of hook and line around Woods Hole.

Because of the comparatively long time which elapsed from the time the fishes were caught in the trawl to the taking of the sample, and because of possible asphyxial conditions in a fish-crammed trawl bag, we extended our work to include also a series of hand-lined fishes. This was undertaken at the Lerner Marine Laboratory at Bimini Island in the Bahamas during December 22 to January 3, 1951. Here the fishes caught down to 220 meters were immediately pulled up by a hand winch, and the gas samples were secured within seven to ten minutes from the strike, faster in shallower water. The depths were measured by marks on the near vertical and heavily loaded line or wire. The data from very shallow water were obtained from captive fishes that had been living in large wire screen enclosures in the sea for at least one week.

It should be pointed out that in most of our deeper fishes, the swimbladder ruptures when the gas expands on the way up to the surface (Fig. 4). In such an event, the gas escapes into the body cavity of the more or less asphyxiated fish and even out through the connective tissue, resulting in spectacular emphysema such as was graphically illustrated by Ledebur (1936). Unprotected by the swimbladder wall, the gas now undergoes more or less rapid diffusion exchange with the body walls. The rate of these exchanges was determined on 6 specimens of ling and cod that had been caught at 4 atmospheres depth and which were lying on the deck. The nitrogen concentration was found to increase by about one volume per cent per ten minutes—*i.e.,* from 25 to 26 volume per cent. The carbon dioxide increased from 0.5 per cent to 0.6 (0.7) per cent. These determinations were made on the half cc. analyzer (Scholander, 1947) and were accurate to within 0.02 per cent. It is therefore likely that all of our nitrogen figures for fishes from deeper waters are from 1 to 2.5 per cent higher than in the living fish at the bottom. There is no apparent difference between the trawled and hand-lined data.

The following fishes were caught and analyzed.

Fishes from the New England Coast

(See Bigelow and Welsh, 1924)

Brosmius brosme	Cusk
Gadus morrhua	Cod
Fundulus majalis	Killyfish
Melanogrammus aeglefinus	Haddock
Menidia notata	Silverside
Merluccius bilinearis	Silver hake
Peristedion cp. miniatum	Deep sea robin
Pollachius virens	Pollack
Pomolobus pseudoharengus	Alewife
Prionotus carolinus	Sea robin
Sebastes marinus	Rose fish
Stenotomus versicolor	Scup
Tautoga onitis	Tautog
Tautogolabrus adspersus	Cunner
Urophyces chuss and tenuis	Ling

Fishes from the Lerner Marine Lab., Bimini

(See Jordan and Everman, 1896–1900)

Acanthurus bahianus	Tang
Apsilus dentatus *	"Black fish"
Balistes carolinensis	Reticulated ocean tally
Balistes vetula	Queen trigger fish
Calamus bajonado	Porgy
Canthidermis sobaco	Ocean tally
Epinephelus guttatus	Red hind
Epinephelus morio	Red grouper
Epinephelus mystacinus	Black grouper
Epinephelus striatus	Nassau grouper
Haemulon album	Margate
Holocentrus ascensionis	Squirrel fish
Lutianus analis	Mutton snapper
Lutianus buccanella	Red snapper
Lutianus vivanus	Long fin red snapper
Melichtys piceus	Black durgon
Ocyurus chrysurus	Yellow tail
Pomacanthus arcuatus	Black angel
Pomacanthus paru	French angel
Seriola dumerili	Amaco jack
Seriola lalandi	Amber jack

* This species was kindly identified by Dr. C. M. Breder, American Museum of Natural History.

The specified data on all of these fishes are presented in Figures 3, 7, and 8. There are in all 16 northern species with samples from 304 specimens and 21 southern species with samples from 80 specimens.

Analysis of swimbladder gas. Immediately after the fish was brought into the boat, it was held under water in a tub and gas was drawn into a 5 cc. syringe tightened with a little glycerine on the plunger and grease on the needle socket. The needle was withdrawn from the fish under water while slowly ejecting gas so as to prevent any sea water from entering the syringe. A small piece of gum rubber cut out of a $\frac{1}{4}$ inch sheet was stuck on to the needle tip under the water.

Airtightness was ascertained by pressing on the plunger while holding the syringe under water. The sample was usually analyzed within a few hours and did not change detectably during 24 hours when analyzed to an accuracy of 0.02 per cent. Due to the considerable biological spread in the data and the error from the unavoidable delay in getting the sample, it was soon found that the analyses could be made much quicker and simpler and still sufficiently accurately by using the capillary syringe (Scholander and Roughton, 1942).

The following method is an adaptation of the original blood gas method (Roughton and Scholander, 1943) which should be consulted for details. With a 50 division capillary syringe, CO_2, O_2 and N_2 can be conveniently and quickly analyzed within 0.5 per cent of the true values, as determined by more accurate methods.

Reagents: Acid solution: Dissolve 70 grams of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in 100 cc. water and add 3 gm. citric acid.
CO_2 absorber: Dissolve 70 gm. sodium citrate in 120 cc. water and add 4 gm. sodium hydroxide.
Oxygen absorber: Add 15 gm. pyrogallol to 100 cc. of 20% NaOH in a rubber stoppered bottle and cover with a layer of oil 2 cm. thick. The pyrogallol is dissolved under the oil by stirring with a glass rod.
Acid rinsing solution: Add 1 cc. concentrated sulfuric acid to 500 cc. water and add 10 mgm. potassium permanganate

The reagents are stored as shown in Figure 1.

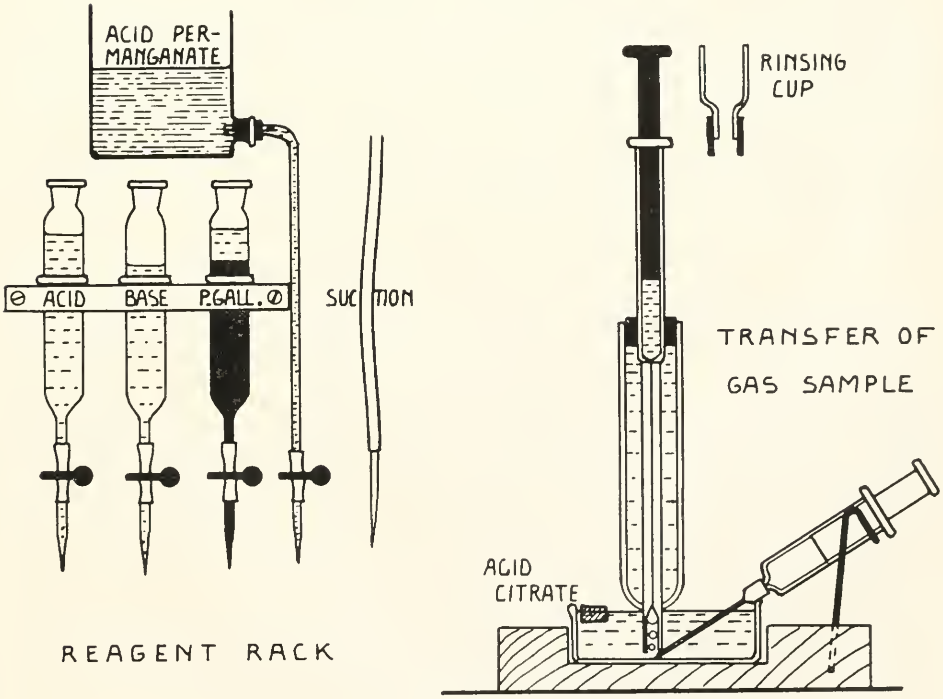


Figure 1. Field method for micro gas analysis.

Procedure. The gas sample contained in the 5 cc. syringe is transferred to the capillary syringe by removing the rubber stopper under the saturated acid citrate solution with a forceps and resting the syringe in a wire holder (see Fig. 1). The capillary syringe, with a water jacket, is filled and rinsed with 0.1–0.2 cc. acid citrate and inverted without trapping air over the needle. The first few gas bubbles from the needle are discarded and the few next ones are caught under the cup of the analyzer and immediately drawn into the capillary. The gas solubility in this almost saturated acid citrate solution is so low that no detectable CO_2 losses will occur before the gas gets into the capillary. The solution has good surface properties for micro gas analysis (Scholander and Evans, 1947). The volume of the gas sample is measured in the capillary by drawing the gas bubble down below the fifty mark and slowly adjusting it back to the zero mark. The CO_2 is absorbed by filling the cup with the alkaline citrate solution and slowly drawing it down over the gas bubble in the syringe barrel, where the absorber layers over the acid. After measuring the gas bubble in the capillary, pyrogallol is filled into the cup and similarly layered over the bubble in the syringe and the gas bubble measured in the capillary as before. At the end of the analysis, the fluid is ejected and is removed by suction. A 2 cc. glass or rubber cup is placed on the syringe cup and is filled with the acid rinsing solution. This is drawn down into the syringe and ejected. The washing is repeated until the permanganate color remains, indicating that all the pyrogallol is oxidized. The acid is again ejected and the cup removed. The dead space of the syringe is rinsed twice with acid citrate and 0.1–0.2 cc. left in the barrel for the next analysis.

Pressure tank. In order to determine at what depth the fishes were in buoyancy equilibrium with the sea water, a conventional nitrogen cylinder was cut off and fitted with a tight screw lid furnished with a lucite window and a pressure gauge. The cylinder was connected with a bucket of sea water through a hydraulic pump. The fish to be tested was put into the sea water filled tank with its head floating free up against the observation window. The lid was screwed on and the water pressure increased until the fish just began to sink. The pressure at which this occurred is called the flotation pressure or flotation depth.

The Nitrogen Tension in the Sea Water

It has been shown by Rakestraw and Emmel (1938) that the nitrogen tension of the sea water is very nearly that of the atmosphere from the surface down to very considerable depths. Hamm and Thompson (1941) obtained similar data from East Pacific waters. From the surface waters (17 degrees) down to 2000 meters depth (1.9 degrees) the nitrogen saturation was found to be 100 per cent ± 3 per cent at 19 out of the 21 different depths. Of a series of 85 water samples taken from 6 different stations in the Atlantic Ocean from the surface down to 4500 meters depth, 82 showed a nitrogen tension within 5 per cent of 0.8 atmospheres—*i.e.*, most of them had almost exactly atmospheric nitrogen tension. Most of these stations showed a considerable change in temperature going from the warm surface waters of 27 degrees to the cold deep waters of only a few degrees. In spite of the very considerable difference in the solubility of nitrogen in these layers, the nitrogen tension was practically constant. This seems indicative of a high degree of stability with regard to temperature, salinity, and nitrogen tension, which characteristics presumably were acquired when these layers were in contact with the surface at some earlier time somewhere. Any subsequent changes in salinity and temperature resulting from mixing of these layers will not change the nitrogen tension of the mixture, because the component layers had the same 0.8 atmosphere nitrogen tension. This, rather than vertical diffusion, may be the reason for the remarkable constancy of the nitrogen tension in the sea water.

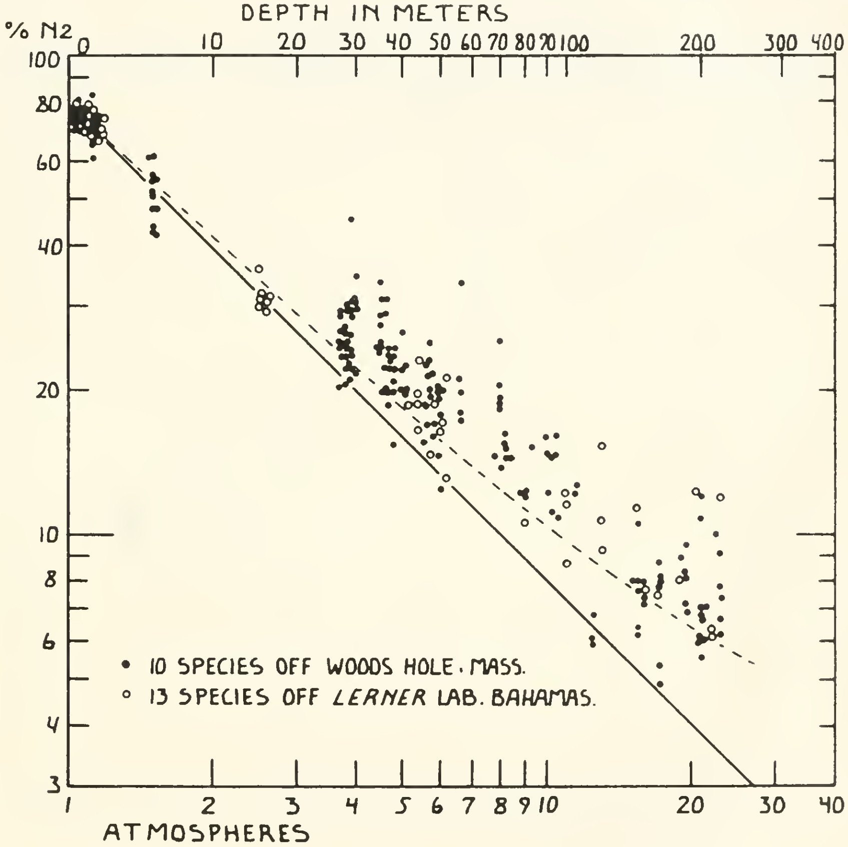


FIGURE 2. Per cent nitrogen in the swimbladder gas of bottom dwelling species in relation to measured bottom depth. The full drawn line represents the nitrogen percentage to be expected if the swimbladder nitrogen were in tension equilibrium with the sea water nitrogen (0.8 atm.). The presented points are uncorrected for post mortem increase known to occur (especially in the deep sea specimens) before the samples can be secured. This amounts to as much as 2½ per cent, and the broken line represents this correction.

COMPOSITION OF THE SWIMBLADDER GAS AS A FUNCTION OF DEPTH

We know from the above determinations that the nitrogen tension throughout the sea water may be taken as 0.8 atmospheres. If we presume that the nitrogen cannot be chemically secreted into the swimbladder, then we would expect it to enter by simple diffusion mostly through the blood stream. Provided the fish stayed at a constant depth, the nitrogen tension in the swimbladder should, therefore, in time, adjust itself to that of the surrounding water—*i.e.,* 0.8 atmospheres at all depths. If such were the case, however, the nitrogen percentage in the swimbladder gas should come out as 80 divided by the total pressure in atmospheres. At one atmospheres pressure—*i.e.,* at the surface—the nitrogen should be 80 per cent, at 10 atmospheres total pressure the nitrogen should be 8 per cent, at 100 atmospheres 0.8 per cent, etc. If the log. of the depth in atmospheres is plotted against the log. of the nitrogen percentage, we would accordingly get a straight line relation such as shown in Figure 2.

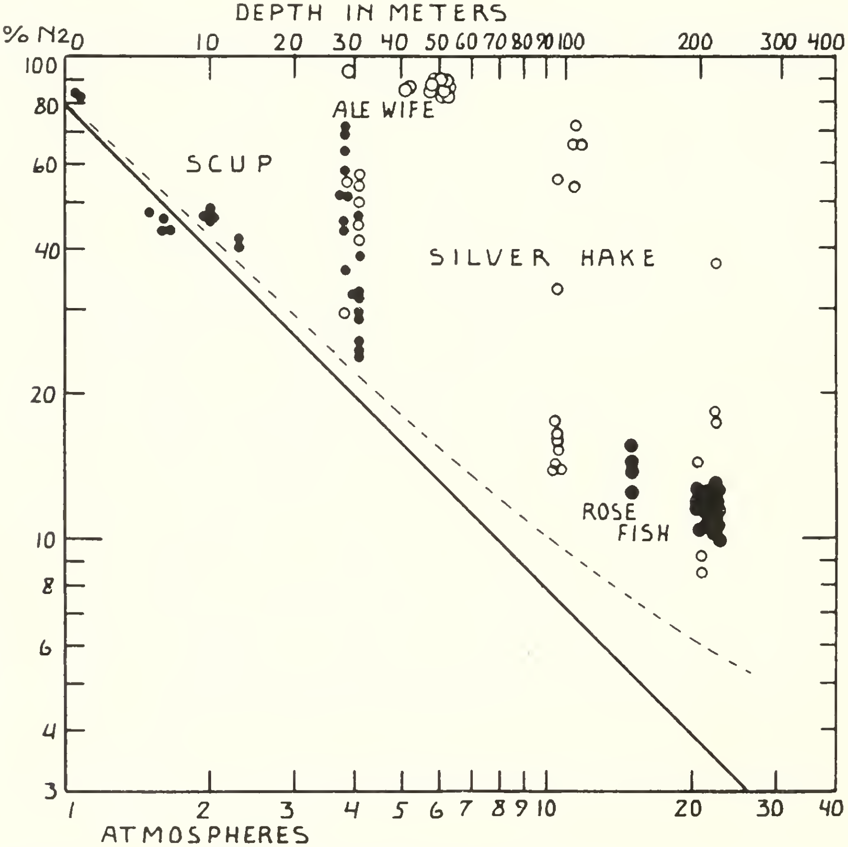


FIGURE 3. Percentage nitrogen in the swimbladder gas of vertically migrating species. The presented points and lines same as Figure 2.

We see in Figure 2 that the predicted correlation between the nitrogen percentage and the depth holds rather well for most of the species. The correlation is even better if we consider that the nitrogen per cent in the intact swimbladder at the bottom was lower than found 5–30 minutes later when the gas was taken from the body cavity of the asphyxiated fish. The dotted line in Figure 2 and Figure 3 indicates how much nearer the points would fit the prediction if the observed values were corrected by subtracting 2.5 volume per cent, or, as is actually done here instead, by adding 2.5 per cent to the predicted line. We may, therefore, conclude that the nitrogen tension in the swimbladder of all those fishes was nearly in equilibrium with that of sea water, and hence is essentially determined by the latter. The CO_2 per cent in all of our samples was rarely above 1 per cent and it is, therefore, clear that the entire pressure work rests on the oxygen secretion (Fig. 6).

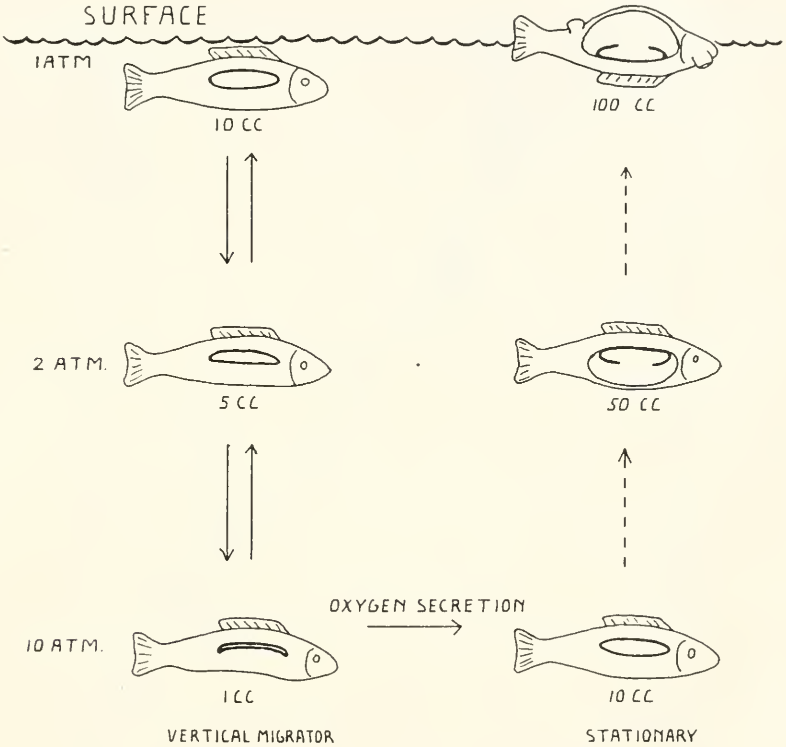
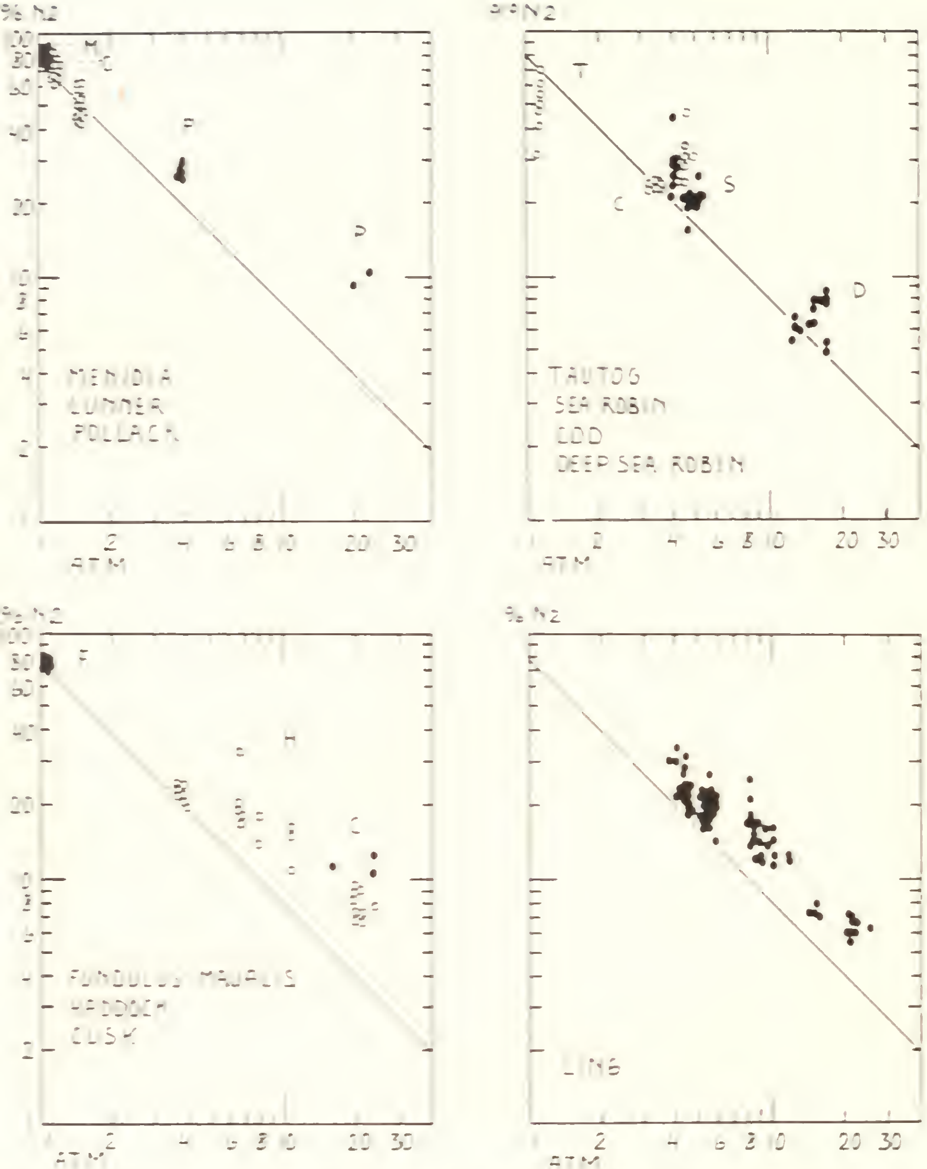


FIGURE 4. Swimbladder gas volume in relation to depth.

The above simple correlation does not hold, however, in all species. The scup, alewife, silver hake, and rose fish are conspicuous exceptions (Fig. 3). In many of these, the nitrogen tension in the swimbladder recalculated to the depth at which they were caught would amount to more than ten atmospheres. How do these high nitrogen tensions come about?

The rose fish is caught at the bottom in many places in great abundance, but when trawled for at night in the same locality it is hardly ever caught. This makes one suspect that they migrate up at night as so many planktonic organisms do. If so, they may acquire a nitrogen percentage characteristic of lesser depth. This depth may conversely be estimated if we know the nitrogen concentration. Corrected for a likely gain of 2 per cent, the nitrogen in our rose fishes caught at about 200 meters depth would be 11 − 2 = 9%. This corresponds to a depth of 80/9



re 7. Relation of nitrogen percentage in the swimbladder gas to the depth in eleven species f fishes off the New England coast.

s were tested in the flotation cylinder, it would be found that the fish would be
oyant at the surface and hence had not secreted gas, and from the surface flota-
n we could hence conclude that the high nitrogen tension was caused by down-
ard migration.

If, on the other hand, the fish is brought down to 10 atmospheres depth and now
ants to regain its lost buoyancy, it must secrete 90 ml. oxygen measured at one

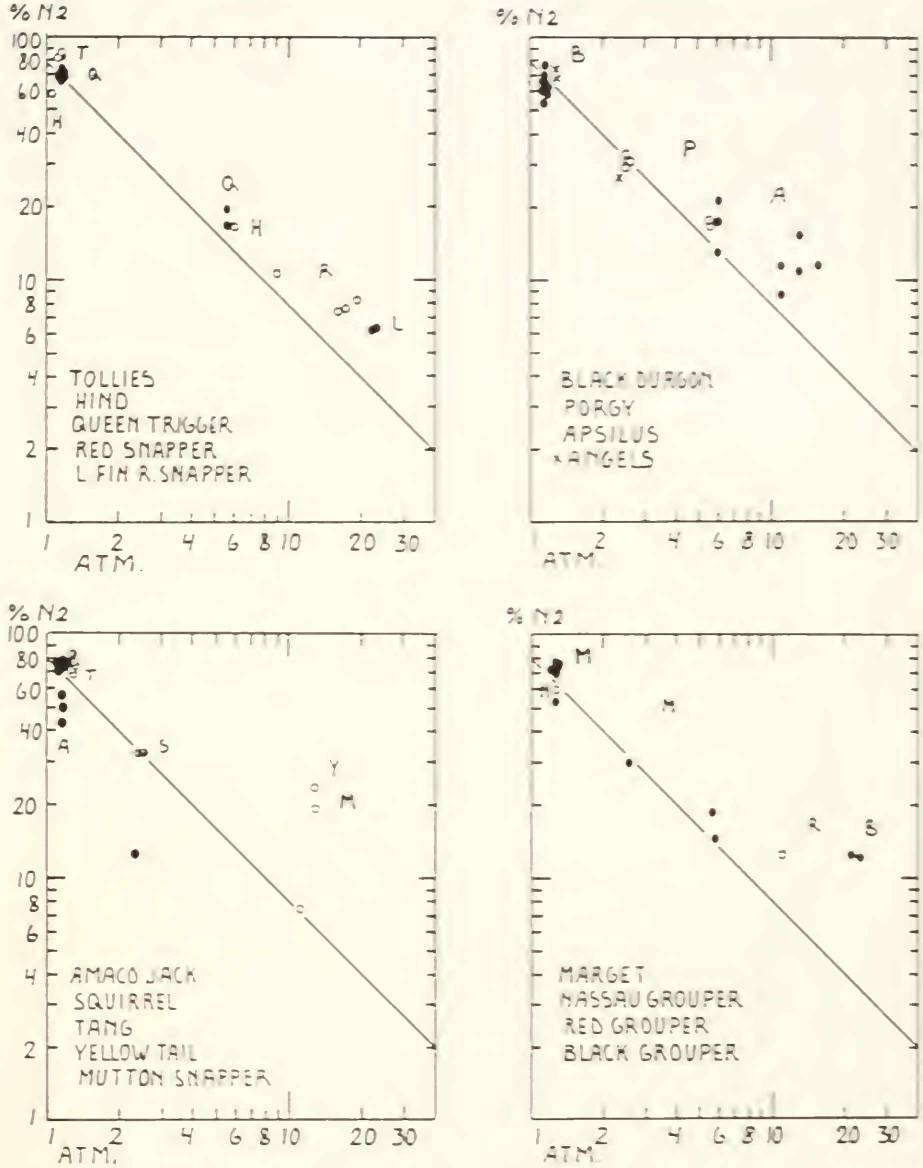


FIGURE 8. Relation of nitrogen percentage in the swimbladder gas to the depth in eighteen species of fishes, taken off the Lerner Marine Laboratory, Bimini, Bahamas.

atmosphere in order to make a total of 10 cc. gas in the swimbladder at 10 atmospheres. This fish is now very much restricted in its upward migration, as any decrease in the hydrostatic pressure will expand the swimbladder gas. Unless the fish has a patent air duct through which to let the gas out (Physostome), it can only very slowly get rid of the gas (Bohr, 1892, 1894).

The expansion of the swimbladder as a function of depth is merely that the

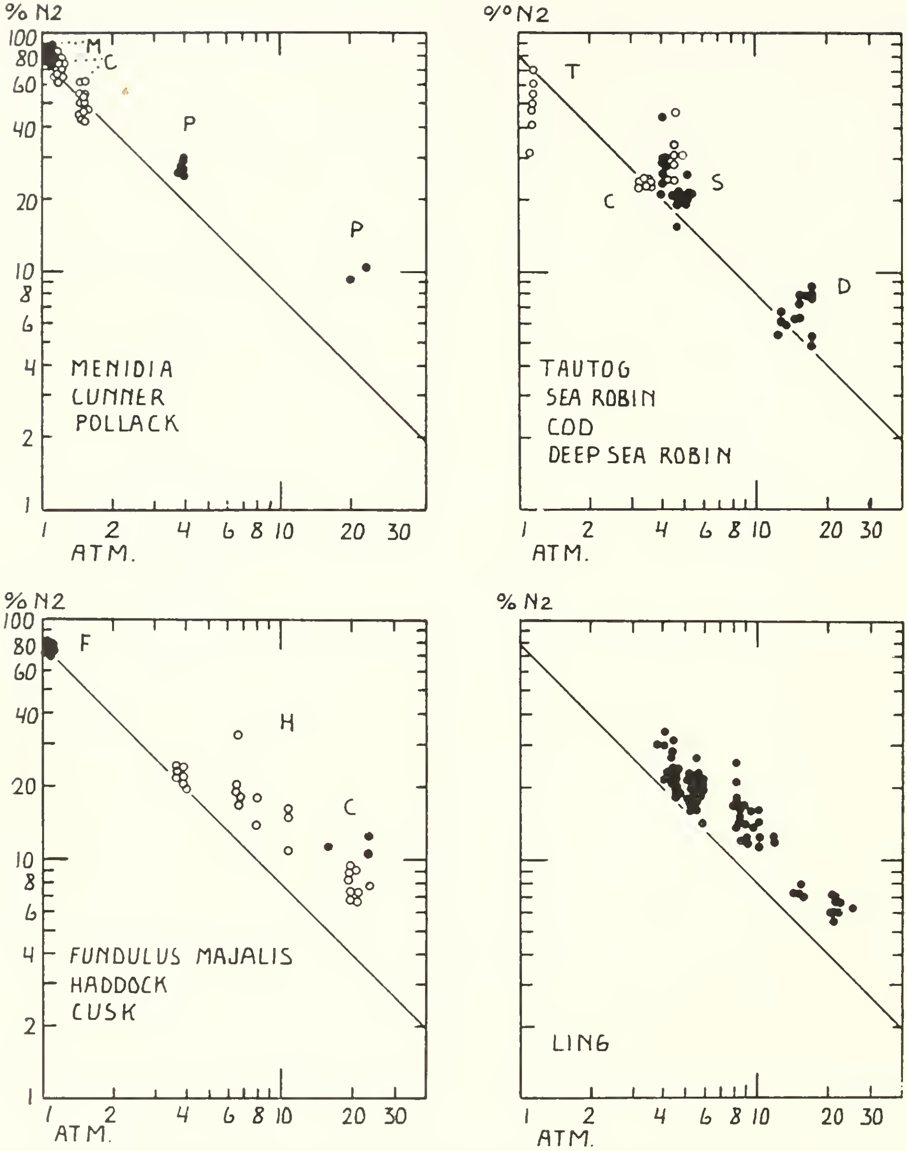
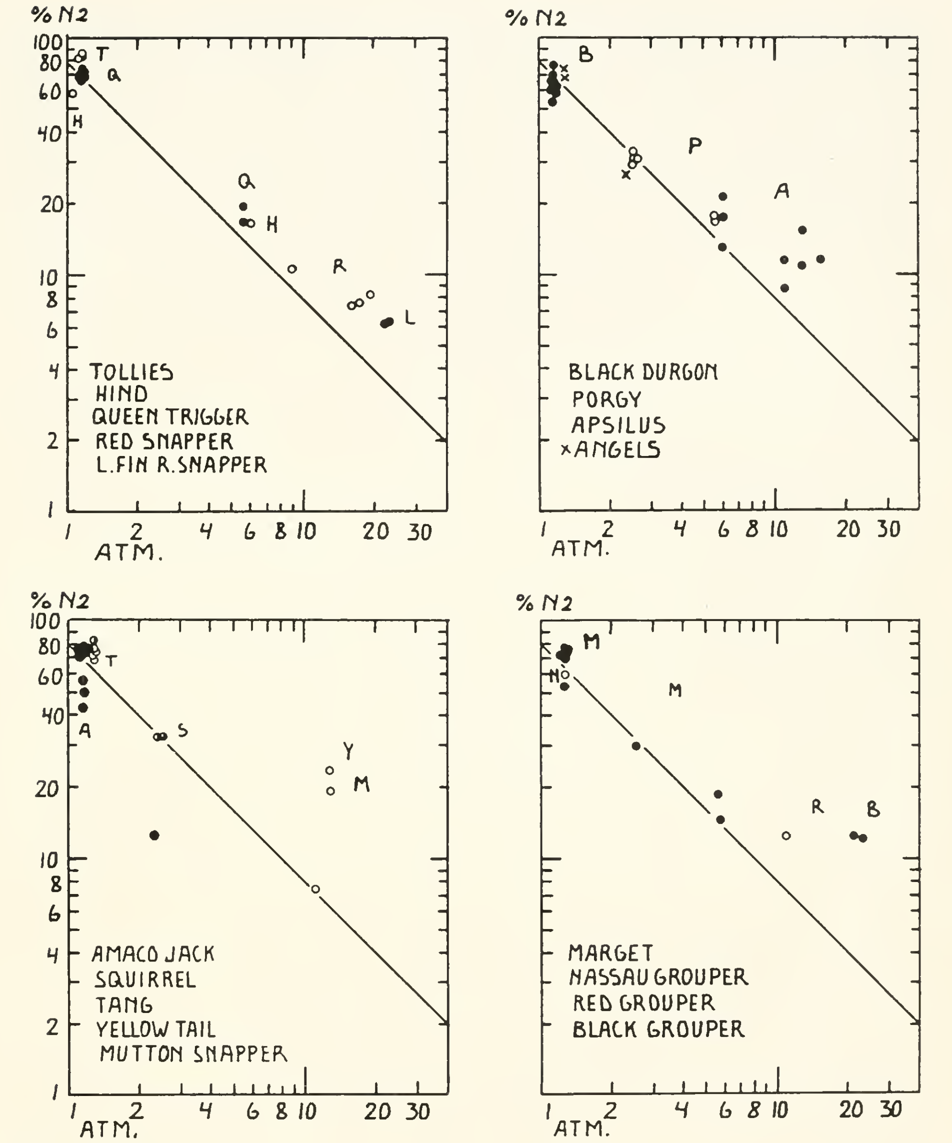


FIGURE 7. Relation of nitrogen percentage in the swimbladder gas to the depth in eleven species of fishes off the New England coast.

fish were tested in the flotation cylinder, it would be found that the fish would be buoyant at the surface and hence had not secreted gas, and from the surface flotation we could hence conclude that the high nitrogen tension was caused by downward migration.

If, on the other hand, the fish is brought down to 10 atmospheres depth and now wants to regain its lost buoyancy, it must secrete 90 cc. oxygen measured at one



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The expansion of the swimbladder as a function of depth is merely that the volume changes inversely to the total pressure (Moreau, 1874). Thus, a fish buoyant at 10 meters depth (2 atm. total pressure) would suffer a swimbladder distension to twice the volume if it were moved to the surface. This, it most likely cannot tolerate. A bottom living fish containing a swimbladder is hence very much restricted in vertical movement and more so, the more shallow it lives. If it can tolerate a 25 per cent distension of the swimbladder, a fish living at 100 meters depth can swim up 22 meters; one living at 10 meters depth can swim up 4 meters; and one living at 2.5 meters can just break the surface. It is doubtful that many species will voluntarily subject themselves to even 25 per cent distension beyond the buoyancy volume. This sort of thing is undoubtedly the reason why so many fishes, observed through relatively shallow waters, so conspicuously refuse to follow the bait up for more than a short distance.

According to these considerations, we may hence conclude that, if a fish has a buoyancy pressure significantly less than the bottom pressure, then its swimbladder is partially collapsed at the bottom and consequently the fish can migrate up for some distance and could hence be in nitrogen equilibration at a lesser depth. If, on the other hand, we find that a fish has a flotation pressure near the bottom pressure, then we know that it must stay there and we could predict that the nitrogen percentage in the swimbladder would correspond to the bottom depth.

We have determined the flotation pressure in several species of fish taken off Woods Hole. Data from depths corresponding to 10 atmospheres or more are of no value, because the fishes leak gas excessively when brought up, thus giving false flotation pressures. The following species that did not seem to lose gas were tested: ling, sea robin, scup, alewife, and silver hake.

In Figure 5, the bottom depth where the fishes were caught is plotted against the flotation depth determined in the pressure cylinder. It will be seen that the lings and the sea robins are pretty well along the bottom—some of them even down in solid rock! The scup, alewife and silver hake, on the other hand, often showed very shallow or even negative depth—*i.e.*, they sank at the surface. These three species were also those that often showed a very high nitrogen figure for the depth. There can, therefore, be little doubt that the high nitrogen tension often found in these species is caused by vertical migration.

These conclusions are supported by what is known about the habits of the fishes we have been working on (Bigelow and Welsh, 1924). The scup is known occasionally to school at the surface. The silver hake and alewife are considered independent of the bottom, often inhabiting the midlevels of the sea. The rose fish, which leak too much gas when brought up for flotation depth measurement, are known to be off the bottom at night (see above), and have been captured in abundance on line trawls suspended at 100 to 200 meters over much greater depths (Murray and Hjort, 1912). Ling, cusk, and sea robin are considered strictly bottom fishes.

We have a few compression data on cod, pollack, and haddock from 30 meters depth. They all seemed to leak gas coming up and had shallow flotation depths although rather good nitrogen values. The cod and the pollack are known to live at any level between the bottom and surface; the haddock rarely leaves the bottom (Bigelow and Welsh, 1924).

Notes on Some Early Observations

In 1896, Schloesing and Richard reported analyses of the gas from the airbladder of the Portuguese man-of-war (*Physalia*) and two deep sea fishes. They determined the argon/argon + nitrogen ratio and found it to be: in air 1.184, in *Physalia* 1.18, in *Murenes* 1.85, and in *Synaphobranchus* 1.94. According to these determinations, the relative argon tension was some 60 per cent higher in the deep sea fishes than in the atmosphere and in the sea water. How this seemingly significant difference has come about we can only guess at; differences in solubility and diffusibility of the two gases are probably involved.

Hüfner (1892) determined the gas composition in the swimbladder of a whitefish (*Coregonus acronius*) netted at 60–80 meters depth in the Bodensee, in the European Alps. When breaking the surface the fishes were strongly inflated, floating on their backs; they could be kept alive only when deflated with a sharp stick (v. Siebold, 1858). Hüfner extracted 120 cc. gas from a 300 gm. fish, which would give a flotation depth corresponding well to the bottom depth. Six out of nine fishes had over 99 per cent nitrogen in their swimbladder. If these data are correct, we have here a case where the 6–8 atmospheres nitrogen tension in the swimbladder cannot have been caused by migration downward. It might seem a possibility that decomposition of organic matters at the bottom might cause the production of such gases as hydrogen, methane, or nitrogen. If this occurred to the extent of bubble formation, the gas tension in the bottom water could become high enough to permit these gases to fill the swimbladder by diffusion. Dr. G. E. Hutchinson, Osborn Zoological Laboratory, Yale University, has kindly furnished us with the following comment on this possibility: "I am prepared to believe that very rarely 1–2 atmospheres of CH_4, N_2, etc. might be so added, but surely not 7–8 and certainly not in the Bodensee in which the O_2 never goes below about 75 per cent saturation at any depth (Auerbach, Maerker and Schmalz, Arch. Hydrobiol., Suppl. 3, 597–738, 1924)." We must leave it to the future to verify Hüfner's data or to find other cases of high nitrogen percentage at deep flotation depths.

Conclusions

According to the present survey based on analytical data from 37 species and 384 specimens, it seems indicated that fishes that live at a constant depth, as along the bottom, acquire a nitrogen tension in their swimbladder gas which is near to 0.8 atmospheres irrespective of the depth—*i.e.,* the same as in the surrounding sea water. They are, hence, essentially in simple diffusion equilibrium with the nitrogen in the sea water. In other species, we find a nitrogen tension in the swimbladder that is often much higher than in the sea water. In these cases we find that the flotation depth of the fishes and their known habits, places them at more shallow depth than the bottom so that the high nitrogen tension found in the swimbladder can be satisfactorily explained by vertical migration. We need not think in terms of "nitrogen secretion" in these fishes. The CO_2 tension in all cases was found to be very low so that practically the total gas pressure in the swimbladder above 0.8 atmospheres is made up of oxygen (Fig. 8).

The present work was made possible by the support of the William F. Milton Fund. We are greatly indebted to Dr. A. Baird Hastings and Dr. Eric G. Ball for helpful advice. We are greatly indebted to Dr. W. F. Royce and Dr. R. J. Buller, U. S. Bureau of Fisheries, Woods Hole, for arranging our participation in the research cruise with M. S. Albatross III and for all the help extended to us on board. Dr. C. M. Breder, Jr., American Museum of Natural History, most helpfully arranged for our work at the Lerner Marine Laboratory, Bimini in the Bahamas. Mr. Michael Lerner gave us the utmost cooperation and free use of his most valuable collection of live fishes. We have had the privilege of cooperation from Dr. I. M. Taylor and Mr. H. H. Woodard who spared no effort in helping us secure preliminary material. Carl Moeller and Johnny Andrews gave most valuable help in constructing our pressure tank.

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ABSTRACTS OF SEMINAR PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

July 3, 1951

The effect of stretch on action potentials in muscle. B. Libet, H. J. Ralston and B. Feinstein.

Supramaximal stimulation of the nerve of an isolated nerve-muscle preparation (frog) produces an electromyogram (EMG) which has a greater amplitude in the stretched than in unstretched muscle. The lower EMG of the unstretched muscle rapidly builds up to the full height of the EMG of stretched muscle if the nerve is stimulated repetitively. This increased EMG due to stretch appears to be due to an increase in the number of muscle fibers responding to the maximal nerve volley (*i.e.*, not all neuromuscular junctions transmit in the unstretched muscle), rather than to any increase in size of the action potentials of the individual muscle fibers, because: (a) no increase in EMG occurs with stretch if the muscle (curarized) is directly stimulated instead of the nerve; (b) the twitch tension developed by an unstretched muscle is greater if the muscle is directly and maximally stimulated than if the nerve is so stimulated; (c) partial curarization makes the facilitatory effect of stretch on the EMG become more evident in the monkey. The role of this facilitatory effect of stretch on neuromuscular transmission in the normal animal is not yet clear.

In normal man or monkey, stretch of muscle *decreases* the amplitude of the EMG whether the muscle is activated by maximal voluntary efforts or by supramaximal shocks to the peripheral nerve. Each individual EMG spike also shows broadening and greater complexity in the stretched muscle; this suggests that a greater average desynchronization of the individual action potentials is produced by stretch (in larger muscles). This would cause the reduction in overall EMG amplitude which might be so great as to mask any increase such as is observed in the frog muscles.

Observations on the development of the slime bacterium, Chondromyces crocatus. John Tyler Bonner, Chambless Johnston and Harold Clifton Urchel.

Chondromyces has in its life cycle an aggregation of bacterial rods which rises into the air to give a small branched fruiting body. The stalk of the fruiting body is composed largely of congealed slime, while the cysts which are borne in clusters at the branch tips enclose large numbers of rods. Upon germination these cysts will split open and liberate the rods which feed and divide, accumulating in larger and larger aggregates until the final fruiting. All these stages were followed with time-lapse motion pictures and numerous novel details concerning their development were revealed. Also some preliminary experiments have been performed on the mechanism which guides the bacteria into the central collection masses and from these it was concluded that the tracks laid down by the previous passage of rods are extremely important in physically orienting the direction of movement of the cells. But also, there is an attraction mechanism which does act at a distance without the help of such tracks. This mechanism could be chemotaxis but there is no positive evidence that such is the case.

An application of ion-exchange resins to the isolation of amino acids from biological material. Leon S. Ciereszko.[1]

With the aid of the ion-exchange resins Amberlite IR-120 and IRC-50, we have been able to isolate, in good yield, and in pure form, the amino acids from rather complex mixtures in which there is one major amino acid component.

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[1] Aided in part by a grant from the Permanent Science Fund, American Academy of Arts and Sciences.

The amino acid was removed quantitatively from the extract or filtered bacterial culture medium by passing the solution through a column of the strong sulfonic acid cation-exchanger Amberlite IR–120. After rinsing the ion-exchange resin with water, the amino acid was brought into solution by displacement with ammonia. Effluent was collected from the time amino acids appeared in the solution. The solution containing ammonia and amino acids from the IR–120 resin was then passed through a column of the weak carboxylic cation-exchange resin Amberlite IRC–50. Amberlite IRC–50 removed the ammonia from the solution, but permitted the amino acids to pass through in solution. The solution from the IRC–50 resin was decolorized if necessary, concentrated by distillation at low pressure, and the amino acid was then crystallized from the concentrate by the addition of alcohol, or, in the case of the dicarboxylic amino acids, by acidification of the solution to a pH value of 2.8–3.0.

Using the method outlined above, we have isolated D-aspartic acid from cultures of *Bacillus subtitis* grown on a synthetic medium containing DL-aspartic acid, asparagine from hot water extracts of tobacco, and glutamic acid, alanine, and methionine from bacterial culture media.

The method as described is not applicable to the isolation of the basic amino acids.

July 10, 1951

Status of the chemistry of Cypridina luciferin.[1] H. S. Mason and E. F. Davis.

In this study an attempt was made to assess the homogeneity of the purest concentrates of Cypridina luciferin available and to test a variety of the structures hypothetically assigned to it. The extremely oxygen-sensitive luciferin was subjected to paper partition chromatography in an atmosphere of oxygen-free hydrogen saturated with the vapor of the developing solvent, and the position of the luciferin was determined by means of light emission in the presence of enzyme. No concomitants could be observed within the limits of sensitivity of the tests employed. However, the luciferin itself separated into two substances, both of which served as light-producing substrates for luciferase. The hypotheses which provide that luciferin is a phenol (Anderson, 1936), a phenolic hydroxymethylketone (Chakravorty and Ballentine, 1941), a naphtholic hydroxymethylketone (Spruit, 1946), a flavin (Eymers and van Schouwenburg, 1937; Johnson and Eyring, 1944), or a compound containing labile phosphate (McElroy and Ballentine, 1944) were not confirmable.

Studies on the metabolism of fetal and placental tissues. Claude A. Villee and Dorothy T. Balzer.

Tissue slices, prepared from fetuses and placentas obtained at therapeutic interruptions of pregnancy, were incubated in Warburg vessels in a Ringer-phosphate medium containing C^{14}-labeled glucose, pyruvate, acetate, oxalacetate or glycerol. Carbohydrate metabolism was studied in the tissue slices and oxygen utilization, substrate utilization, and glycogen, carbon dioxide, and lactic acid production were measured. Fourteen fetuses ranging in age from nine to about twenty-three weeks were used. It is difficult to make comparisons between different experiments, for not only are the fetuses of different ages and genetic stocks, their mothers had different diseases. Yet the metabolism of these tissues is not dissimilar; the tissues from fetuses of diabetic mothers behave as normal, not as diabetic.

It was possible to observe the development of the enzyme glucose-6-phosphatase: it appears at about 11 weeks in the liver and at about 16 weeks in the kidney. Although the fetus has been reported to be insulin-fast, the addition of insulin *in vitro* produces an increase in glucose uptake, glycogen formation and pyruvate and lactate production in diaphragm, heart and kidney, as in the comparable adult tissues.

By as early as the eighth week of development, the fetal tissues are well differentiated biochemically and show the patterns of carbohydrate metabolism characteristic of the corresponding adult tissue. Liver has a high glycolytic rate, brain has the highest rate of glucose utilization and the lowest rate of pyruvate utilization of the five tissues studied: brain, heart, diaphragm, liver, and kidney. The heart has the highest rates of pyruvate utilization and lactic acid production. The tissues show a wide variation in initial glycogen content, from 27 mg. per gm. wet weight in liver to 0.16 mg. per gm. in brain.

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[1] Work supported by the Research Corporation, New York, and the Eugene Higgins Fund of Princeton University.

The diaphragm rapidly metabolizes pyruvate to CO_2, heart metabolizes more pyruvate than glucose to CO_2 and the liver metabolizes about equal amounts of the two. Brain metabolizes pyruvate to CO_2 well but uses acetate only slowly. In contrast, liver metabolizes acetate at least as well as pyruvate. Oxalacetate is decarboxylated rapidly by all tissues and the presence of unlabeled oxalacetate decreases the amount of radiopyruvate metabolized to CO_2. The oxalacetate is apparently decarboxylated to pyruvate and thus dilutes the radiopyruvate present

Auxin as the hormonal factor normally limiting the differentiation of xylem cells around a wound. W. P. JACOBS.

The hypothesis expressed in the title has been confirmed in the following ways: Auxin shows a strong polarity of movement in Coleus internodes, whether one tests the auxin naturally present or adds synthetic auxin. The strong basipetal movement of auxin is paralleled by a strong basipetal polarity of xylem regeneration; the relatively slight acropetal movement of auxin is paralleled by a slight acropetal differentiation of xylem. Removal of all the leaves and buds below the wound has no effect on xylem regeneration—this is expected since it has been shown that these leaves produce relatively small amounts of auxin and that only a small percentage of the amount produced can move up the stem. Removal of the leaves and buds above the wound markedly reduces the amount of xylem regeneration, the amount of reduction varying with the number of auxin sources removed. A concentration of synthetic auxin such as normally comes from Leaf No. 2, the major auxin source, completely replaces the xylogenic effect of Leaf No. 2. Finally, auxin has been shown to be normally present in very small amounts in Coleus internodes; hence, it can conceivably be acting as a limiting factor.

JULY 17, 1951

Polarographic investigation of the reaction between organic mercury compounds and thiols. REINHOLD BENESCH AND RUTH E. BENESCH.

The importance of thiols in biological systems has led to the wide use of organic mercury compounds for their detection and characterization since these compounds are relatively specific in their interaction with -SH groups, in contrast to divalent mercaptide-forming metals such as cadium or copper.

Polarography provides a specific and sensitive method for the quantitative assay of organically bound mercury, which has been lacking hitherto. This method was therefore used to study the reaction of two classes of organic mercury compounds, *i.e.*, phenylmercuric compounds and mercurated allylamides (used in medicine as diuretics), with mono- and dithiols.

(1) Reactions with Glutathione: The organic mercury compounds investigated form stable mercaptides with glutathione which can be detected and determined polarographically since they are reduced at different potentials from the free mercurials. Moreover, it is possible to determine the glutathione mercaptides and the free mercury compounds in the presence of each other.

(2) Reactions with 2,3-dimercaptopropanol (BAL): The reaction of phenylmercuric compounds with BAL is stoichiometric and leads to the formation of an insoluble dimercaptide. The reaction between the mercurated allylamides and BAL leads first to the formation of an unstable intermediate product. This can be identified as the dimercaptide by working at 0° C., where it is sufficiently stable to permit its polarographic characterization. This product decomposes at room temperature with the formation of an insoluble precipitate. Polarograms of the supernatant solution showed that the mercaptide waves had disappeared completely and free

organic mercury compound had reappeared. The quantitative relationships are in agreement with the following reaction mechanism:

$$CH_2OH\cdot CHSH\cdot CH_2SH + 2\,Hg^+RCOO^- \rightarrow CH_2OH\cdot CHSHgRCOOH\cdot CH_2SHgRCOOH \rightarrow$$
$$\rightarrow CH_2OH\cdot CH\underbrace{S\cdot CH_2SHg}_{} + Hg^+RCOO^- + HRCOOH$$

The rate of the progressive reaction is greatly increased by an increase in temperature. It is also accelerated by increasing hydrogen ion concentrations, which is undoubtedly related to the ease with which the C—Hg bond in the mercurated allylamides is split by acid. The conversion of the diuretic molecule into an insoluble cyclic mercaptide by BAL provides an explanation for the suppression of mercurial diuresis by this compound.

Blood and liver lipids in the northern pike, Esox lucius. CHARLES G. WILBER.

Practically all the work on lipids in fish has been restricted to investigations on marine forms. It was therefore decided as part of an overall program of arctic biological research to study the lipids in various Alaskan freshwater fish. The northern pike, Esox lucius, taken from Lake Minchumina was selected as one of these. The blood lipid values in mg. per 100 cc. are: total fatty acids, 771; cholesterol, 183.3; phospholipids, 411.6. The liver lipid values in per cent fresh tissue are: total fatty acids, 9.1; cholesterol, .89; phospholipids, 3.1.

A comparison of these values with those for various marine fish shows that there is appreciably less fat in the liver of the northern pike than in that of many marine fish, where total oil values of as high as 60 per cent are found. Blood fatty acids and cholesterol are not remarkably high, but the phospholipids are in greater concentration than in the blood of various animals such as man, dog, or rabbit.

The phospholipid value in the liver is much like that in the livers of many poikilothermes and of some homothermes. On the other hand the cholesterol concentration of close to 1 per cent in the liver is noteworthy. Whether it is all cholesterol or whether some other sterol is included was not ascertained. It is known, however, from previous work that the chief sterol in fish is cholesterol.

In view of the high liver cholesterol, it is suggested that further intensive studies of the liver of the northern pike be made. A complete fractionation and identification of the sterol complex is indicated. As a practical matter careful attention should be paid to the vitamin content of these livers.

Calcification in molluscs: The localization of Ca^{15} and P^{32} in the mantle and regenerating shell. GERRIT BEVELANDER.

Previous observations have shown that the formation of the shell in molluscs consists in the formation of a protein matrix known as the periostrachum, and subsequently the mineralization of this membrane by calcium carbonate.

The periostrachum is elaborated at the distal part of the mantle. One obvious function which can readily be ascertained is the role it plays in supporting and binding the crystals into various layers or strata which make up the shell. Less obvious, but of considerable interest is the fact that the periostrachum is a fairly complex protein containing several constituents which may play an active role in the mineralization of the shell.

In the course of several experimental procedures we have observed that Ca^{45} and P^{32} are taken up by the mollusc when these substances are placed in water in trace amounts. Subsequent examination of the mollusc reveals the presence of Ca^{45} on the periphery of the mantle. The P^{32} was located on the inner surface of the mantle which we have also identified as a region which is rich in alkaline phosphatase. Radio-autographs of regenerated shell, grown in the presence of Ca^{45} and P^{32} show Ca^{45} to be localized in the crystals of the shell; P^{32} is confined to the protein membrane surrounding the crystals.

Our experiments have demonstrated that calcium and phosphate in ionic form are readily ingested from the water; further, these ions are concentrated in the mantle and various other organs, and finally both Ca^{45} and P^{32} are eventually deposited in the crystalline and organic parts of the shell respectively.

The localization of phosphatase in the mantle, in the region of the mucus glands containing organic phosphate, suggests a parallelism between this correlation and that found in developing bone. In the case of shell formation, however, the final calcified product is calcium carbonate, while in bone the calcified substance is a phosphate complex (apatite). Whatever role alkaline phosphatase may play in the process of calcification, it appears unlikely that it is concerned with a simple hydrolysis to make available phosphate ions to be incorporated into the mineral component of calcified tissues—either bones, teeth or shells.

JULY 24, 1951

Influence of direct current on the long axis distribution of K, Ca and Na in dog nerve. SIDNEY SOLOMON AND JULIAN M. TOBIAS.[1]

Earlier work has led us to suspect that ultrastructural changes in nerve occurring as part of the excitation process, whether at stimulating electrodes or as part of the process of self-excitation (conduction), are caused or allowed by particular changes in some critical ion ratio, as K/Ca. We have suggested that due to mobility differences Ca and K may be separated along the nerve long axis by the potential existing between stimulating electrodes and also by the potential existing between a now-active and an about-to-become active locus. If so, then on a measurable scale, one might find that a sufficient and relatively prolonged voltage applied to nerve would move both K and Ca toward a negative region, but would move K there more rapidly thus increasing the local K/Ca ratio, opposite changes occurring anodally. Such polar-peculiar changes in this ratio might lead to alterations in ultrastructure such as dispersion and agglomeration which could be responsible for the trans-surface impedance changes known to accompany excitation.

Therefore, dog sciatic nerves have been excised and then polarized on wick or metallic electrodes for varying times with varying voltages, and have been subsequently sectioned into 5 mm. bits along the long axis. These bits were then analyzed for K, Ca and Na.

It has been found that the K/Ca ratio increases at a cathode and decreases anodally. Sodium moves cathodally as expected, but the evidence suggests that some of the Ca actually moves toward the anode, leading tentatively to the conclusion that at least some of the Ca in nerve is bound to a particle, the whole complex being negatively charged.

Influence of various potassium concentrations on the oxygen consumption of frog nerves. RUDI J. H. OBERHOLZER.[2]

In order to determine the oxygen uptake by excised frog sciatic nerves, bathing in Ringer's solution containing various potassium concentrations, a flow respirometer has been used. The rates of oxygen consumption thus observed have been compared with the resting rates measured for each nerve bathing in frog Ringer at pH 7.1 and 20° C.

In solutions with 20 *mM* KCl a 5% increase of the oxygen consumption has been observed over a period of 4 hours. In solutions containing 40 *mM* KCl the increase of the resting respiration reached a maximum of +14% in ½ hour, then the oxygen uptake gradually decreased, attaining after 4 hours a new steady level of −10%. In a bathing solution of 120 *mM* KCl, where all sodium has been replaced by potassium, the rate of oxygen consumption showed in the first ¼ hour an average increase of +20%. This short-lasting phase was followed by a period of depressed respiration with an average reduction of −30% from the second hour on. The effects were reversible. These results may explain some contradictory observations reported by previous investigators.

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[1] This work has been aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago, and in part by a grant from the United States Public Health Service, Panel on Genetics and Morphology.

[2] Fellow of the Rockefeller Foundation for Medical Research and Public Health.

Reaction of substrates and inhibitors with the surface of acetylcholinesterase. I. B. WILSON. No abstract submitted.

The curare effect and axonal conduction. D. NACHMANSOHN. No abstract submitted.

JULY 31, 1951

Chemotropism in Rhizopus nigricans: The staling reaction. DAVID R. STADLER.

This mold, like many others, exhibits oriented growth away from dense populations toward regions of more sparse growth. This behavior (the "staling reaction") is exhibited when all physical factors in the environment are known to be homogeneous; thus it must be a response to chemical heterogeneity.

The earlier finding has been confirmed that the staling reaction is oriented growth away from a metabolic product of the mold (the "staling substance"), not toward higher concentrations of some nutrient.

Attempts to further substantiate the chemical nature of the staling substance by showing its presence in cell-free filtrates have failed. A culture with a heavy inoculum is allowed to grow; then the mold is filtered off. Spores inoculated adjacent to this staled filtrate grow into it as readily as into fresh medium. Thus the staling substance is very unstable. Other experiments indicate that it is not highly volatile.

Experiments with various aged cultures show that staling substance concentration increases at about the same rate as protoplasmic mass.

Concerning the mechanism of turning, the hypothesis has been tested that tropic growth results from differential growth rate across the hypha caused by unequal distribution of a limiting growth factor produced by the mold itself. Thus the staling substance would itself be a diffusable growth factor causing hyphae to grow faster on the side nearer the heavy population and turn away from it. Checks of linear growth rate in heavy and sparse cultures bear out the hypothesis—molds do grow faster in heavier culture. But tests with the cell-free, staled medium show that this medium, which fails to exhibit active staling substance, still does accelerate growth. Thus the growth factor and the staling substance appear to be separate factors.

A second hypothesis—that the staling substance acts by suppressing cell wall enlargement in its presence—has been substantiated by preliminary experiments.

Observations of the flight mechanism of flies. EDWARD G. BOETTIGER AND E. FURSHPAN. No abstract submitted.

Specificity of steroids which inhibit heart beat.[1] IVOR CORNMAN.

Inhibition of pulsation in roller tube cultures of chick heart and mouse heart by desoxycorticosterone was reported earlier. Progesterone also slowed or stopped most chick heart fragments at 0.01 mg/ml and blocked all pulsation at 0.02 mg/ml within 1–3 hrs. Testosterone at 0.02–0.03 mg/ml. (undissolved crystals present) induced partial inhibition at 12–24 hrs. Estradiol in suspension at 0.03 mg/ml, or as a large excess of crystals in the tube had no effect on pulsation, nor did estrone or dehydroisoandrosterone acetate as an excess of crystals. Prolonged exposure to progesterone at 0.02–0.03 mg/ml damaged the fibroblasts. In the presence of an excess of testosterone crystals, the fibroblasts eventually rounded up, but exposure for several days in tubes heavily sown with estradiol, estrone, or dehydroisoandrosterone acetate crystals had no visible effect on these calls. The soluble steroids desoxycorticosterone glucoside up to 0.1 mg/ml and potassium estrone sulfate up to 0.2 mg/ml did not inhibit the pulse during

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[1] Supported by an American Cancer Society institutional grant to the George Washington University Cancer Clinic and by a grant-in-aid from the Washington Heart Association.

2-day exposure. A synthetic progestational non-steroid, 1,2-*bis*-(*p*-aminophenyl)-2-methylpropanone-1-dihydrochloride, perceptibly inhibited pulsation at 0.04 mg/ml and blocked it at 0.1 mg/ml. Within this series, substances with progestational activity were the most effective in stopping heart pulsation, whereas other steroids, whether soluble or not, showed little activity.

AUGUST 7, 1951

The nature of the hemolytic action of dodecyl ammonium chloride. WARNER E. LOVE.

The interaction of a typical surface-active cation, dodecyl ammonium chloride (DACl), and human erythrocytes has been investigated by studies of the type of hemolysis produced by it. DACl produces hemolysis by changing the erythrocyte in such a way that it becomes freely permeable to cations. Evidence for this mechanism of hemolysis is provided by the following experimental results:

a. With a newly developed optical method for the separation of swelling from hemolysis, it has been shown that human erythrocytes in a physiological salt solution swell prior to hemolysis by DACl.

b. In solutions of DACl in 0.3 *M* sucrose the cells shrink to a very small volume before they swell and eventually hemolyze.

c. On the addition of a small amount of NaCl to a suspension of such cells in sucrose, they at first shrink very rapidly and then recover their volume more slowly, *i.e.*, NaCl behaves as a typical penetrating substance.

d. DACl increases to a remarkable degree the permeability of erythrocytes to normally non-penetrating non-electrolytes.

Like surface-active anions, this cationic substance protects erythrocytes from its own hemolytic action. Conditions have been found where desorption of the agent from the cell by dilution of the medium with 0.9% NaCl or by changing the pH toward acidity causes immediate and rapid hemolysis.

The effects of both the ionic strength of the medium and the time of exposure to the agent prior to desorption have been studied. Increasing the ionic strength of the medium and increasing the time of exposure both favor the protective action of DACl; it is therefore proposed that something akin to micelle formation occurs at the surface of the cell, the result of which is either to prevent the escape of hemoglobin or to reduce the degree of induced permeability to cations, or both.

The relation between hemolysis and the potassium content of red cells. A. K. AND E. R. PARPART AND T. DEY.

The standard curve of osmotic resistance of the red cells of normal rabbits is very reproducible from animal to animal. The red cells go from no hemolysis at 0.6% NaCl (phosphate buffered in all cases) to complete hemolysis at 0.4% NaCl. Red cells of rabbits were exposed to tonicities in this range at dilutions of 1 to 40. After 1 hour equilibration the environment of the cells was made isotonic with NaCl, and the unhemolysed cells were packed in an air turbine. These packed cells were analysed for K and Na. Over the hemolysis range from 0 to 85% the K and Na content of the unhemolysed cells was found to be the same.

It is necessary to point out that microscopic examination of these unhemolysed red cells reverted to their original volume showed no "ghosts" among the packed cells. This was not true for the unhemolysed cells which were packed in the swollen state.

It is concluded that the osmotic, pre-hemolytic swelling of red cells does not involve a loss of K or a gain of Na by these cells, over 1 hour of exposure to the hypotonic environment. It is further concluded that at least 85% of the red cells in a given population have practically identical K content, and it is suspected that this is true of the entire population.

Some microchemical reactions of the erythrocyte. M. H. JACOBS.

Erythrocytes are chemical systems of very constant composition whose constituents are measured in millionths of a millionth of a gram. As such they can be employed for a variety of chemical tests. Cases have been described elsewhere in which the erythrocytes of certain species show a special sensitivity to silver and to copper, respectively, and can be used to detect traces of these elements.

Further uses of erythrocytes as microchemical reagents are provided by a group of reactions in which swelling and hemolysis in solutions of NH_4Cl give visible evidence of the presence of various specific substances in low concentrations in the surrounding medium.

By taking advantage of the "catalysis" of the entrance of NH_4Cl by bicarbonates it is possible to estimate very small quantities of the latter and also carbon dioxide tensions. In the presence of known amounts of bicarbonates, means are provided for the assay of the enzyme carbonic anhydrase and its inhibitors. Low concentrations of so-called indifferent narcotics can be estimated by their ability to retard NH_4Cl hemolysis. An even stronger inhibitor is tannic acid. Very small quantities of it and of substances which neutralize its inhibiting effect such as organic bases and proteins can likewise be measured by the NH_4Cl hemolysis method.

A method of possible practical usefulness for the estimation of urinary ammonia is provided by the characteristic swelling of erythrocytes exposed to ammonium salts in a suddenly alkalinized medium. By photoelectric-photographic recording, concentrations of the order of 1×10^{-5} *M* can be measured in this way. Less delicate, but practically useful, are other methods employing an ordinary photoelectric colorimeter or even no equipment but common laboratory glassware and the naked eye.

AUGUST 14, 1951

The occurrence of twinning in Fundulus heteroclitus after centrifugation. J. R. SHAVER.[1]

Twinning occurs in *Fundulus heteroclitus* following centrifugation of eggs for 30 mins. × 293 g, beginning 10 to 30 minutes after fertilization. This observation was confirmed, twinning occurring in 0.75% of eggs centrifuged under these conditions. Two hundred eggs were isolated immediately following treatment, and the complete developmental history of two cases of twinning was described; both were cases of complete twins (no fusion of main axes), but cases of anterior duplication and "parasitic" twins have also been observed. From 37.3 to 72.8% of eggs centrifuged during the period of blastoderm formation failed to develop embryos, and 18–52% of the survivors displayed abnormalities. Embryos with skewed axis were the commonest abnormality found (29.7% of abnormal isolates). Deficiencies in mesodermal structures (no somites, reduced number of somites, unilateral somite deficiency, reduced trunk) characterized many embryos; it is thought that these deficiencies were due to irregularities of the germ-ring seen in many of the isolated embryos. Embryos centrifuged in later stages (2-cell, many-cell, gastrula, early embryo), although displaying characteristic "skewing" of cleavage pattern or embryonic axis, did not develop into twins, and the percentage of normal embryos resulting approached that of the controls. It was suggested that in Fundulus the period of formation of the blastodisc is especially sensitive to disorientation of materials that might represent a "primary organization center"; if it is assumed that cortical (or subcortical) materials which form the blastodisc early establish physical contact and rates of reaction with underlying substrata, necessary for normal embryogenesis, the results of the centrifugation might be interpreted as disturbing these relationships.

Significance of the periblast in teleost gastrulation. J. P. TRINKAUS. No abstract submitted.

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[1] Aided by Summer Research Professorship, University Research Council, University of Missouri.

Specific fertilizin agglutination of dead sperm. CHARLES B. METZ AND JOANNE DONOVAN.

Properly prepared dead sperm should be useful for the study of the role of the sperm surface in fertilization. Ideally the sperm should be killed and fixed without appreciable alteration of the specific molecular organization of the cell surface.

To prepare such material washed living sperm were treated with the killing agent and when necessary washed again by centrifugation or dialysis. The dead sperm were then tested for agglutination with homologous fertilizin. Since fertilizin agglutination is a highly specific reaction, this test should provide a fair index of the effect of the killing agent on at least one sperm surface group.

Arbacia sperm, which were examined in most detail, failed to give satisfactory agglutination after killing with most agents. However, sperm treated with alcoholic Bouin's fixative (equal volumes of sperm and 25% to 50% fixative) agglutinated strongly on addition of fertilizin. Furthermore, the fixed sperm paralleled living sperm with respect to agglutination by a number of other agents (Asterias egg water; Nereis, Echinarachnius, Mactra fertilizins; Arbacia and Asterias bloods; heparin; univalent Arbacia fertilizin). Fixed and living Arbacia sperm differed in ability to agglutinate in Mactra tissue fluid. However, the dead sperm agglutinin in this material was absorbed by living sperm. Thus the reactive groups for the Mactra agent are present on both living and fixed sperm. Apparently, then, Arbacia sperm retains its specific surface organization to a very considerable degree when treated with Bouin's fixative.

Experiments to determine if amino acid side groups are essential for dead sperm to agglutinate with fertilizin indicate that phenolic and sulfhydryl groups are not essential but suggest that amino groups must be free at the sperm surface for the sperm to agglutinate.

AUGUST 21, 1951

Cooling as a stimulus to smooth muscles. JOHN F. PERKINS, JR. AND WILLIAM H. JOHNSON.

We have quantitatively studied the effects of rapid and slow cooling alone as a stimulus causing contraction of various mammalian smooth muscles and of frog intestine when cooled below 10° C. Rapid and slow cooling act as graded stimuli, rapid cooling being the more effective, as noted by Starling in 1912.

When degrees of rapid cooling are plotted against heights of isotonic contraction, one obtains an S-shaped "cooling-response" curve which falls off with extreme amounts of cooling. That this falling off is not due solely to an increased "viscosity" or internal mechanical resistance preventing shortening is shown by the fact that the same degree of falling off occurs with isometric recording. It appears more likely, then, to assume that with extreme cooling less energy is released, either for shortening, or for developing tension.

Several lines of evidence indicate that cooling alone acts on a different system than epinephrine. For example, after Dibenamine, which reverses the action of epinephrine so that the latter now tends to produce lengthening, cooling still causes a contraction. Normally, the separate effects of cooling and of epinephrine are additive when both are applied simultaneously, but after Dibenamine the two stimuli tend to cancel (algebraic addition, epinephrine having minus sign).

Recent studies indicate that increased amounts of potassium ion potentiate the action of cooling alone as a stimulus, shifting the cooling-response curve to the left. Whether cooling alone will act as a stimulus to the smooth muscles of marine fauna, with and without additional potassium, is being investigated.

It is hoped that an assault upon the excitation-conduction-contraction systems in smooth muscles may be facilitated by using cooling alone as a stimulus, in conjunction with a method developed by us (1950) for studying the viscous-elastic properties of smooth muscles.

Studies in carbohydrate and lipid metabolism, using C^{14}-labelled glycerol. M. L. KARNOVSKY.[1]

Radio-glycerol should be a useful tool in the elucidation of the pathways of lipid metabolism—especially in studies on the biosynthesis of phosphatides. Since glycerol may be considered an offshoot of the glycolytic cycle, the use of radio-glycerol should yield interesting data on lipid-carbohydrate inter-relationships.

C^{14}-labelled glycerol has been synthesized, and metabolic experiments carried out on rats. The labelled compound was administered in normal saline, by intraperitoneal or intravenous injection, or by intragastric intubation, and the animal was immediately placed in a metabolism train. The expired CO_2 reached its peak activity in about 70 to 90 minutes, and the curves were apparently independent of the mode of administration of the glycerol. After 6 hours the animal was sacrificed, and the organ lipid and carbohydrate fractions examined. About 50% of the administered counts had been expired, and about 5% of the administered counts were to be found in the liver lipids. The phosphatides were significantly more active than the neutral fats.

In one series of experiments animals were dispatched at different times after administration of the radio-glycerol. The blood sugar and liver-glycogen reached peak activity at about one hour, and at this peak the amount of liver glycogen derived from glycerol was about 15–20%. The amount of administered glycerol that had then gone to glycogen was about 5%.

Experiments have also been carried out with triglycerides labelled in the glycerol portion of the molecule. The most striking feature has been the decrease in the ratio of the activity of the phosphatides to that of the triglycerides in the case of triolein. Tributyrin behaved essentially like free glycerol.

A number of studies have been carried out *in vitro,* in which rat liver slices have been presented with radio-glycerol, sometimes together with non-radioactive pyruvate. These experiments, together with their converse experiments, have led to some interesting data on the behaviour of the DPN-linked glycerol-dihydroxyacetone: pyruvate-lactate system.

A selective mechanism in Escherichia coli. LILLIAN K. SCHNEIDER AND HELEN CROSS.

Cultures of a histidineless mutant (h −) of the bacterium *Escherichia coli,* serially transferred for long periods of time in medium containing optimum histidine, show a gradual increase in the number of histidine independent (h +) cells, until an equilibrium ratio of 1.3 h + per 10^6 h − is reached. This ratio has been maintained in one experiment for more than 3100 generations. Mutation pressure alone is not sufficient to account for this equilibrium level since the mutational equilibrium, predicted from independent measurements of the forward and back mutation rates, is 1 h + per 100 h −. Specific selection against h + was postulated.

The periodic replacement of the entire population by mutations conferring a selective advantage upon the bacteria in which these changes occur has offered an explanation of the specific equilibrium reached. When a mutation to a fitter type occurs, all other mutant types that have arisen in the population are simultaneously eliminated. Since in our experiments there are from 10^4 to 10^6 times as many h − as h +, the advantage mutant occurs in the h − component first. This fitter h − now becomes predominant in the population and eliminates the previous h − and h +, resulting in an apparent specific selection against h +.

At least two of the steps to successively advantageous mutations are due to intrinsic growth rate differences in the medium in which selection has occurred, suggesting that the growth rate difference is medium-dependent. However, this explanation does not hold in all cases and some steps may involve interactions between different components in the population.

Periodic selection thus could act to maintain the stability of a bacterial population in an environment to which it is suited. At the same time it acts to preserve the bulk of the genotype while allowing for variability in a new environment.

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[1] All the experiments described were carried out with the collaboration of Mr. L. I. Gidez and Dr. C. T. Teng.

GENERAL SCIENTIFIC MEETINGS

August 27–28, 1951

Drugs inhibiting growth and nucleic acid synthesis in Arbacia embryos. Dorothy T. Balzer and Claude A. Villee.

Mathews (1904) and Sollman (1906) had shown that development was retarded by atropine and pilocarpine, and previous work (Villee *et al.*, 1949) had shown that dinitrophenol, malononitrile, uranyl nitrate and nitrogen mustard, which inhibit cleavage, also inhibit the synthesis of ribonucleic acid (RNA), desoxyribonucleic acid (DNA), or both. The effects of atropine, acetyl choline, eserine and pilocarpine on development and on phosphorus metabolism in developing Arbacia eggs were studied using radiophosphorus. Embryos were incubated 7½ hours in 100 ml. of sea water containing the drug and 3 to 5μC of P^{32}, then homogenized in ice-cold 7% trichloracetic acid and fractionated as in the previous (1949) experiments.

All drugs tested showed an acceleration at low concentrations, 5 mg. per 100 ml., and an inhibition at higher concentrations, 60, 100, or 500 mg. per 100 ml., of growth and phosphorus metabolism, in the order eserine > pilocarpine > atropine > acetyl choline. Eserine affects the enzymes responsible for the uptake of phosphorus into the acid-soluble P fraction of the cell; it has little or no effect on phospholipid P metabolism. Its most marked effect, the one most closely correlated with the effects on cleavage, was its inhibition of the incorporation of P^{32} into the DNA P fraction. These experiments do not indicate whether the action of eserine is on some part of the cleavage mechanism and DNA synthesis is inhibited secondarily, or whether the eserine acts on some phase of DNA synthesis and cleavage is secondarily retarded for lack of DNA. The metabolism of RNA phosphorus is unaffected, even by 100 mg. of eserine per 100 ml., at which concentration DNA P incorporation is only 16 per cent of the control value. Pilocarpine also showed a marked inhibition of the incorporation of P^{32} into DNA but no inhibition of RNA metabolism.

A new DNA fraction of the cell nucleus. Jay Barton II. See abstract in "Reports on Lalor Fellowship Research."

Hemolysis of sheep erythrocytes by organic mercury compounds. Ruth E. Benesch and Reinhold Benesch.

Organic mercury compounds of the phenylmercuric and of the mercurated allylamide type have been found to be actively hemolytic towards sheep red cells. Two per cent suspensions of sheep erythrocytes were mixed with the organic mercury compounds under isotonic conditions and shaken gently at 37° C. Aliquots of the suspension were removed at definite time intervals. The intact cells were separated immediately by centrifugation and the extent of hemolysis was measured by colorimetric determination of the hemoglobin in the supernatant fluid. The phenylmercuric compounds caused hemolysis in lower concentrations than the mercurated allylamides. Thus half the cells were lysed in 28 minutes by 5×10^{-4} *M* phenylmercuric hydroxide, while it took 100 minutes to lyse half the cells with 8×10^{-4} *M* Mersalyl. The rate of hemolysis was found to vary enormously with slight changes in the concentration of both types of organic mercury compound. It was further demonstrated that interaction of the organic mercury compounds with an equimolar amount of glutathione, which results in the formation of organic mercury mercaptides, abolished the hemolytic effect of both types of organic mercury compound. These findings suggest that the lytic properties of these compounds are based on their interaction with —SH groups essential for the maintenance of the intact cell structure. Since it has been shown that the red cells of certain fish are lysed by extremely minute concentrations of silver and inorganic mercury ions, it is planned to investigate the effect of organic mercurials on such cells and to correlate it with the concentration of —SH groups in these cells.

Propagated changes in light scattering accompanying activity in nerve.[1] SHIRLEY H. BRYANT AND JULIAN M. TOBIAS.

Little or nothing is known of the changes in protoplasmic ultrastructure (molecular-macromolecular) which may accompany activity in nerve. Toward the detection, measurement and understanding of such components of function, experiments have been designed which yield measurements of changes in light scattering by nerve which accompany impulse propagation. To date, many of our findings have been similar to those of Hill (1950). However, in addition we have found changes in active frog nerve, and the temporal course of the changes seen has been much different in our experiments as compared to those of Hill.

The white light scattered at right angles to an incident beam impinging on a 2 mm. length of frog, crustacean or squid multi-fibered nerve or squid giant axone has been measured using a balanced photocell arrangement.

In essence, what one observes is that the passage of a burst of impulses is accompanied, in some cases, by an initial, small, short-lived increase in scattering. More constantly, with or without this initial increase, there is a more delayed, longer lasting and larger decrease in scattering which, in turn, is followed by recovery more or less to the resting condition. In the frog sciatic only the decreased scattering phase has been observed. Conditions upon which the type and duration of changes seen in the other nerves depend are in process of being worked out. However, hypotonicity of the medium has been found to favor the appearance and duration of the early phase of increased scattering.

Failure of the changes to appear with just sub-threshold stimulation or with supra-maximal stimulation in non-conducting nerves, and lack of dependence on electrode orientation and distance of stimulating electrodes from the light region, rule out current spread from the electrodes as the causative agent.

Naively dividing the maximal change in total scattered light by the number of impulses shows, for the decreased scattering phase, a change of from 0.000006% in the frog to one of 0.01% in the crustacea, per impulse.

Experiments to determine the precise time course and meaning of the changes are in progress.

Autogamy of Paramecium aurelia (stock 51.7) grown on non-living media. W. D. BURBANCK AND MADELINE P. BURBANCK.

Experimental animals were obtained from Dr. T. M. Sonneborn. These were grown on the non-spore-forming bacterium, *Aerobacter aerogenes*, grown on lettuce infusion and in the "two anlagen" stage of autogamy were washed free of bacteria according to Parpart's technique. Washed animals were placed individually on a non-living medium consisting of a 1.5 cc. culture made up of 1.0 cc. *A. aerogenes*-lettuce infusion (with the bacteria killed by exposure to 60° C. for 10 minutes) and 0.5% yeast autolysate (original Basmin-Busch by Anheuser-Busch). Following the first post-zygotic fission, one of the progeny was used to start a caryonide. In 24 hours, eight animals were produced. They were each placed in 1.5 cc. cultures of the following media with the indicated results:[2]

(1) Control grown on living *A. aerogenes* went into autogamy on average of 4.5 days.

(2) *A. aerogenes* used as food was killed by exposure to 60° C. for 10 minutes. Animal placed on this medium died in 24 hours with macronuclear disintegration.

(3) Like (2) but 0.5 cc. of 0.5% yeast autolysate added. Progeny of this animal regularly (seven runs) went into autogamy in 4.5 days.

(4) Bacteria killed by autoclaving (15 lbs., 15 minutes) and 0.5 cc. of yeast autolysate added. Some animals showed macronuclear fragmentation or hemixis.

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[1] This work has been aided in part by a grant from the United States Public Health Service, Panel on Morphology and Genetics, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] *Aerobacter aerogenes*, living or killed, used in all eight media. In first four, it was grown on lettuce infusion and on "Cerophyl" infusion for last four. Except for (2) and (5), all *P. aurelia* progeny produced each day were transferred until 64 were obtained and these were then allowed to reproduce without further transfers.

(5) Like (1) but went into autogamy in 4.0 days. This was the Cerophyl control.
(6) Like (2).
(7) Like (3) but less regular, animals went into autogamy twice in five runs.
(8) Like (4).

Heat death in Phascolosoma. ALFRED B. CHAET.

Heat death, according to the "toxic factor" theory, is the result of a toxic substance which is first found at the site of the burn and is later dispersed throughout the animal's body. Although there have been many studies on heat death and the effect of burns on vertebrates, very little work has been done on the lower animals. The present work was undertaken in an attempt to determine whether or not similar toxic substances could be found in heated invertebrates. For this study an unsegmented annelid, *Phascolosoma gouldi*, was chosen. Heating the posterior half of Phascolosoma (for 90 seconds in 76° C. sea water) causes death in about 16 hours. The animal's life is prolonged for approximately 73 hours by ligating the animal before scalding, thereby restricting the major part of the toxic factor to the posterior end of the worm. Additional evidence for the presence of a toxic factor is given by an experiment in which cell-free coelomic fluid is taken from scalded animals and injected into healthy animals. Such an injection causes death within 24 hours, whereas injections of fluids from normal animals have no effect. The toxic factor is heat stable as well as non-dialyzable. It is very soluble in water, slightly soluble in absolute methyl alcohol, but not soluble in ether. Although the toxic coelomic fluid has a lower pH than the normal, the toxic effect is not lost when the pH is readjusted to that of the normal coelomic fluid. By equalizing the potassium content of the toxic and normal fluids, it can be shown that the toxic effects described here are not due to potassium. Preparations of the clam heart, *Venus mercenaria*, have been successfully employed as test material for the toxic substance.

The physical state of the axoplasm in situ in the nerve of the squid mantle. ROBERT CHAMBERS AND CHIEN-YUAN KAO.

It is already known that the axoplasm of the giant nerve of the squid possesses, under normal conditions, a gel-like rigidity which, under both dark and light field illumination, appears glass-clear and structureless. Evidence of a linearly arranged ultra-structure has been inferred from the fact that drops of inert oils and air bubbles, when microinjected *in situ* within the neurilemma, assume ovoid shapes. Tests were made to ascertain the degree of rigidity of the axoplasmic gel within various regions of the axon from its center to the periphery. It was found that aqueous solutions of acid dyes which diffuse readily when injected give definite evidence of a relatively fluid core. When microinjected, the solution takes a preferred route of running along the central core through the nerve. The dye then diffuses radially until the entire axoplasm becomes uniformly colored. When oil or air is injected the ovoid shapes assumed differ according to the site injected. At the center the drops are broad oval. As the site of injection approaches the periphery the drops become narrower until, in the extreme cortical zones, the drops are narrow cylinders. The ends of the drops are always ovoid. All these regions are readily liquefied by the introduction of $CaCl_2$ in concentrations of this salt in sea water. Axons, extirpated from the squid mantle with the cut ends tied off with silk threads, give, when immersed in sea water, no indication of the calcium liquefying action except at the bases of the open cut ends of side branches. All these regions are readily liquefied by the introduction of $CaCl_2$ in concentrations of this salt in sea water.

The internal hydrostatic pressure of the unfertilized activated Fundulus egg exposed to various experimental conditions. ROBERT CHAMBERS, EDWARD L. CHAMBERS AND CHIEN-YUAN KAO.

Eggs which had reached the constant internal hydrostatic pressure of 150 mm. Hg were immersed in varying concentrations of sea water. When the external medium was hypotonic to sea water the pressure rose within three minutes to relatively high values and then almost immediately fell to the original pressure of 150 mm. Hg. A similar change but in the reverse direction occurred when the external medium was hypertonic to sea water.

These experiments indicate a rapid passage of electrolytes through the chorion with the establishment of equilibrium conditions between the electrolytes of the external medium and those of the perivitelline space. To account for the pressure which is maintained at the same value irrespective of the electrolytic concentration of the external medium there is to be inferred the existence of a non-diffusible substance which remains constant in the completely formed perivitelline space. Fertilized eggs in which the blastodisc had completed its formation were freed of the enveloping chorion. The isolated egg cells exhibited no change in diameter irrespective of the concentration of the sea water in which they were immersed. This indicates either that the electrolytes in the yolked egg are combined so as to be osmotically inactive, which is improbable, or that the external surface of the egg, at least in this particular stage, is impermeable to water and to electrolytes.

Behavior of cortical granules in methylated xanthine effects. Ralph Holt Cheney.

It is accepted generally that there is a critically essential relationship between the normalcy of the clear, vacuolar-like, cortical granules of the Arbacia egg and normal fertilization. Certain concentrations of di- and trimethylxanthines interfere with the fertilization and development of *A. punctulata.*

To investigate the effect of these compounds upon cortical granules, eggs were pretreated in various molarities of caffeine, theobromine, and theophylline in sea water for different time intervals at the temperature of running sea water (22° C.). Examination of fertilization sequences were made by adding sperm to a hanging drop culture via a micropipette and the visible changes observed continuously at 1100 × under light or dark field illumination until the fertilization membrane formed and was freed completely from the egg surface. Time events were recorded with a stop watch. Normal and centrifuged eggs were employed.

Fertilization was successful and time relationships remained equivalent to the controls as long as the concentration of methylated xanthines, or the immersion period, was insufficient to cause a change in the clear cortical granules prior to the introduction of sperm. Non-fertilization was predictable when the fine, uniform distribution of the cortical granules was seen to be interrupted in part by the apparent disappearance of some of the granules and an irregular grouping into several masses of the majority of these granules.

This change in the physico-chemical behavior of the granules under the influence of methylated xanthines corresponded synchronously with the inability of the egg to be fertilized. The nature of the effect of all three compounds was identical. The intensity of the effect was one of degree in the order of decreasing effectiveness $C > Tb > Tp$ for equivalent molarities.

Urea adduct formation as a means of separating components of lipid mixtures. Leon S. Ciereszko, M. L. Karnovsky and Ann Brumm.

Bengen discovered in 1940 that hydrocarbons and their derivatives form crystalline adducts with urea. The structural requirement for urea adduct formation is a sufficiently long carbon chain with little or no branching. The urea adducts are insoluble in organic solvents, and may be decomposed by treatment with water.

Urea adduct formation has been applied to the separation of fatty acids, but not to the fractionation of the non-saponifiable matter of fats.

We have found that cholesterol and squalene do not form stable urea adducts, while glyceryl ethers, such as batyl alcohol, do. Material containing 90–100% of glyceryl ethers has been recovered from the urea adducts obtained from the non-saponifiable matter of shark liver oil, and of the pyloric caeca fat of starfish. Saturated glyceryl ethers may be separated from the unsaturated glyceryl ethers as the saturated compounds react with urea preferentially.

Enzymes of a ribose-forming pathway during the early embryonic development of Chaetopterus. Seymour S. Cohen. See abstract in "Reports on Lalor Fellowship Research."

Alleviation of the anti-mitotic effect of rotenone by alpha tocopherol.[1] IVOR CORNMAN AND EDWARD F. ROGERS.

To analyze the nature of inhibition of Arbacia egg division by rotenone and similar compounds, chemically related naturally occurring compounds were tested for antagonistic effects. Rutin, folic acid and isorhamnetin were without effect on cleavage delay or suppression induced by rotenone or tephrosin. Alpha tocopherol at 0.01 ml/L partly or completely reversed the rotenone effects. A delay of 65 minutes induced by 0.004 mg/L rotenone was reduced to ten minutes and the ten minute delay resulting from exposure to 0.002 mg/L was abolished by alpha tocopherol. Oils which lack vitamin E activity (mineral oil and olive oil) were without effect on rotenone inhibition. Cottonseed oil, which has vitamin E activity, had less effect than pure alpha tocopherol. It is proposed to test whether the anti-rotenone effect of alpha tocopherol is related to its vitamin E activity or to its anti-oxidant activity by testing other tocopherols. It appears significant at this point that colchicine and podophyllotoxin are antagonized by inositol, which, like vitamin E, has lipotropic properties. One possibility is that lipid metabolism supplies energy or metabolites essential to cell division in the sea-urchin egg. Another is that lipotropic agents provide an auxiliary energy source which the cell can use when normal pathways are blocked by mitotic poisons. The latter is supported by the antagonism of glucose for colchicine and podophyllotoxin. The pathways blocked by these poisons are not identical. Podophyllotoxin, which acts cytologically like colchicine and not like rotenone, is not antagonized by alpha tocopherol.

The movement of the cortical layer of Arbacia eggs in Ca-free medium through cleavage and the resting stage, as revealed by the shift of the pigment granules. KATSUMA DAN.

Previous studies on the movement of kaolin particles adhering to the outer surface of the Astriclypeus egg and also on the change in the cross-sectional width of the differentiated cortex of the Strongylocentrotus egg indicated that at the completion of the first cleavage, the cortical material accumulates around the base of the "stalk" between the two blastomeres. This material spreads and thins out along the surface during the resting stage, pushing most of the pre-existing cortical material toward the polar region. Immediately before the second cleavage, however, the thinned-out surface contracts again, pulling back the polar cortex and bringing about a more uniform distribution of the cortical material.

In *Arbacia punctulata*, many pigment granules are caught in the cortex at fertilization and serve as landmarks for the movement of the cortical layer. Soon after the first cleavage, the pigment granules accumulate densely around the stalk. Within 12 minutes (at 24° C.) the furrow region becomes practically colorless, the pigment granules all shifting in position toward the pole, where they pile up. Eight minutes later, or at 21 minutes after the beginning of the first cleavage, a tendency is perceived for the granules to move back toward the furrow. The granule density on the cell surface continues to become uniform until the second cleavage, which occurs at 24 minutes. Similar movement is repeated for the next cleavage cycle, although a complete redistribution becomes harder as the number of the blastomeres increases, *i.e.*, some pigment-poor area is left on the furrow side when the next division comes.

Cortical movement of this type is now well established, since the landmarks both outside and inside the cell show the same thing and, moreover, it is found in different forms of sea urchins.

Isotonic solutions for fish erythrocytes. JAMES W. GREEN AND JOSEPH F. HOFFMAN.

In a previously reported study on isotonic NaCl solutions for red cells of mackerel, tautog and smooth dogfish, it was shown that the densimeter technique of Parpart provided an accurate and rapid means for determination of isotonicity when compared with other methods. The method records the degree of swelling or shrinking of red cells in salt solutions of various tonicity. Using this technique the red cells of a number of the common Woods Hole fish were studied.

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[1] Aided in part by an American Cancer Society institutional grant to the George Washington University Cancer Clinic.

With teleosts the isotonic values depended upon the method of obtaining the sample of blood. With blood derived from the heart, the values for 14 different species ranged from 0.105 M for shark sucker to 0.262 M NaCl for mackerel, with the majority under 0.2 M. Blood from cut gills for 8 separate species ranged from 0.157 M for flounder to 0.274 M NaCl for tautog. The results with heart blood are thought to be closer to the true tonicity since the gill blood is probably contaminated with sea water.

For 5 different species of elasmobranchs isotonicity values ranged from 0.30 M for smooth dogfish to 0.38 M NaCl for the tiger shark. In all cases the blood was collected from either the heart or caudal vessels with no differences observed. In addition to NaCl some artificial solutions listed in the *MBL Formulae and Methods III* were studied. Knowlton's solution was found to be hypotonic; the "Elasmobranch" solution indicated an equivalence to 0.325 M NaCl; Van't Hoff's artificial sea water was equivalent to 0.38 M NaCl.

The lytic action of calcium ions on the pigment granules of the Arbacia egg.[1] PAUL R. GROSS.

Suspensions of Arbacia egg pigment granules can be prepared by homogenizing the eggs in the absence of free calcium. If calcium is then added to the homogenate, the granules break down, releasing a red pigment (echinochrome). This reaction is believed to be of importance for the life of the cell.

Since the lysis of the granules is accompanied by a color change, the reaction can be studied colorimetrically. By centrifuging the homogenates at high speeds (17,000 × g. for 10 min.) it is possible to obtain a clear supernatant fluid which contains the color. The absorption spectra have been studied and the most convenient wavelengths for the analysis determined.

The rate of granule lysis, but not the final percentage of granules lysed, depends upon the initial concentration of calcium. The reaction thus resembles hemolysis by surface-active agents. Relatively minute (10^{-3} M) amounts of calcium are required to initiate the reaction. Strontium and magnesium also induce lysis, but higher concentrations of Sr are needed, and still greater quantities of Mg ions. Ether, chloroform and soaps give the reaction in the absence of calcium.

When pigment granules are isolated, and washed 8–10 times in isotonic KCl, breakdown will no longer occur under the influence of Ca. Permitting the homogenates to stand for several hours at room temperature causes a sharp decline in the sensitivity to Ca. These observations agree broadly with those recently made upon the granules of *Paracentrotus lividus* by Hultin (1950). The latter worker's suggestion concerning the existence of a soluble, enzymic co-factor is further supported by the following: In the Arbacia material, the reaction is inhibited by Cu^{++}, Hg^{++}, cystine, and organic mercury compounds. Cyanide does not inhibit the reaction. There is a possibility that the co-factor is a protease.

Incorporation of deuterium into proteins of Arbacia eggs suspended in heavy water. HENRY D. HOBERMAN AND CHARLES B. METZ.

Fertilized and unfertilized Arbacia eggs were suspended in 10% deuterium oxide made isotonic with sea water. At designated times eggs were removed from the medium, preserved in alcohol, and later extracted with 5% trichloroacetic acid and with alcohol and ether. The water resulting from the combustion of this fat-free material was then decomposed to hydrogen. The deuterium content of this hydrogen was then determined by mass spectrometry. It was found that for the first 3.5 hours the rate of incorporation of deuterium into the proteins of fertilized eggs was only slightly greater than for unfertilized eggs. After this period the rate of incorporation of deuterium into the proteins of fertilized eggs approximately doubled. This acceleration was coincident with formation of the Arbacia blastula.

The maximum isotope concentration (C_m) which would be reached after complete turnover of the egg proteins was calculated. The value so obtained was equivalent to 10% of the deuterium concentration of the medium. Thus at least one out of every 10 stably bound hydrogen atoms would be a deuterium atom at the time of complete turnover of the egg proteins. This suggests that in the blastula, proteins are synthesized from amino acids rather than from peptides. If the latter had been the case, the value for C_m would have been far lower than calculated. The data obtained in this experiment indicate that the half time of turnover for the proteins of the fertilized egg is approximately one day.

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[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

Some differential permeability effects of n-butyl alcohol. M. H. JACOBS.

It has previously been shown that n-butyl alcohol in sufficiently high concentrations induces in the erythrocyte a condition of cation permeability which may be regulated with considerable exactness by the concentration employed and the duration of the exposure. Under the conditions chosen for study the cells always undergo swelling followed by hemolysis.

More recently Ponder has reported that subhemolytic concentrations of butyl alcohol increase the permeability of the erythrocyte to potassium without causing it to swell. Though in several respects the two cases are not strictly comparable, it should be noted that swelling of a cation permeable cell might theoretically be opposed by a simultaneous reduction by the butyl alcohol of its normal anion permeability. That such an effect may in fact be present over a wide range of concentrations is shown by the enormous retardation by butyl alcohol of hemolysis in solutions of ammonium salts of strong acids whose entrance into the erythrocyte requires anionic exchanges, but not in those of weak acids where a molecular mechanism of penetration is involved.

A simultaneous appearance of hemolysis associated with cation permeability and protection presumably associated with reduced anion permeability can be demonstrated in erythrocytes treated with butyl alcohol in a solution of Na_2SO_4 to a point considerably short of hemolysis and then transferred to a solution of a sodium salt having a more rapidly penetrating anion, *e.g.*, NaCl. Erythrocytes so treated hemolyze rapidly, but the hemolysis may be checked by suitable sub-hemolytic concentrations of butyl alcohol. It is possible, though not yet proved, that the anti-hemolytic effect of certain other hemolytic agents may in part depend upon a similar principle.

Measurement of viscosity and elasticity of smooth muscles during contraction. WILLIAM H. JOHNSON AND JOHN F. PERKINS, JR.

"Viscosity" (mechanical resistance) and elasticity have been measured in the smooth muscles of Phascolosoma in a study aimed at clarifying the relation between the excitation and contractile systems in smooth muscles. An improved version of the apparatus previously described by us was used. The principle involves an analysis of the harmonic motion of one end of the muscle when a sinusoidal vertical motion is imparted to that end of the muscle through a weak spring. Our primary objective consists of a comparison of the viscous-elastic changes in a smooth muscle when activated by different stimuli including electrical, neurohumoral, ionic and cooling alone as a stimulus. Preliminary studies indicate that there are comparable increases in the viscosity and the elasticity of the pharyngeal retractor of the Phascolosoma with tetanic electrical stimulation on the one hand and with stimulation with excess potassium on the other hand. Our ultimate objective consists of measurement of the frequency and temperature dependence of viscosity and elasticity, in the hope of increasing our knowledge of the molecular changes occurring during contraction.

Rates of feeding and oxygen uptake in some filter feeding marine invertebrates. C. BARKER JØRGENSEN. See abstract in "Reports on Lalor Fellowship Research."

The internal hydrostatic pressure of the unfertilized Fundulus egg activated by puncture. CHIEN-YUAN KAO, ROBERT CHAMBERS AND EDWARD L. CHAMBERS.

These eggs, when stripped from the female and placed in sea water, spontaneously undergo activation until the blastodisc is formed. When punctured the activation is greatly accelerated. The unactivated eggs exhibit minute spherical vacuolar bodies, the platelets, lying on the surface of the eggs underneath the chorion which is tough and non-elastic made evident by the fact that the chorion resists volume changes whatever the salt content may be of the environment up to that of sea water. Activation consists of the disappearance of the platelets concomitant with the appearance of a progressively widening perivitelline space between the chorion and the egg cell proper. The diameter of the egg cell decreases in proportion to the increase in width of the perivitelline space. The internal hydrostatic pressure of the eggs was determined by a mercury manometer connected through a column of water in a glass tube to a micropipette the tip of which, containing a short column of oil, was inserted into the egg under view through the microscope. The column of oil was maintained at the tip of the pipette by varying the pressure in the glass tube through the use of a 2 cc. Luer syringe connected at right angles to the glass tube. The pressure required to maintain the oil at the tip of the pipette was recorded by the height of the mercury in the manometer which, at the start, with the pipette tip in air, was 0 mm. Hg, *viz.*, one atmosphere.

The experiments consisted of inserting the tip of the pipette into an egg as soon as the egg was transferred from the mother into sea water. Within 3 minutes the pressure was found to be − 5 mm. Hg, indicating that the eggs were responding to being transferred to sea water which is hypertonic to the normal body fluid of the mother. As the platelets disappeared with the formation of the perivitelline space, the pressure progressively rose during the following 10–20 minutes until it reached a value of 150 mm. Hg which persisted during the following 60 minutes and longer. Positive pressures were obtained only in those eggs which had been activated, with the disappearance of the platelets and the formation of the perivitelline space. When the chorion is snipped open the fall of pressure indicates that the pressure is due only to conditions within the intact perivitelline space.

Metabolism of α-glyceryl ethers in marine forms. M. L. KARNOVSKY. See abstract in "Reports on Lalor Fellowship Research."

Vital staining by fluorescent dyes. R. KELLER.

Electric fields were investigated qualitatively by vital staining. The method used is built up on staining of test objects of uncontroversial electrotopography; the accumulation of dyes in biological objects of known fields gives a strong clue as to the distribution of electric charges in unknown fields. Conclusions drawn from studies of this kind have been supported by a long series of mineral analyses, determinations with electrometer and micromanipulator and with one-dimensional paper chromatography. Intracellular (basic) dyes are mostly travelling in aqueous solutions of high concentration in filter paper and in living protoplasm to the cathode, in dilutions of above 1 : 50,000 to the anode in both media. In diseases the whole intracellular (biologically positive) group diminishes in blood plasma and increases in organ parenchyma; the intracellular group was found to do the contrary. These changes may be taken as a characteristic consequence of the breakdown of electric potentials. Experiments with mice and chicken embryos were taken in partnership with S. S. Wanderman, who classifies fluorescent dyes in one-phasic and diphasic. The latter stain differently, dependent on their electric charge; for instance, thioflavin S is blue at strongly positive and yellow-green at strongly negative structures. Kodachromes were demonstrated of gall bladder, whose walls appeared strongly negative, of incubated chicken eggs with preponderantly negative embryos, of mouse cancers with negative growing neoplasms, with negative injury currents. The slides illustrate the possibility of applying dyes for the investigation of the otherwise invisible microscopic distribution of the electric charges of different tissues.

Iodine studies on Ascophyllum using radioactive iodine. SALLY KELLY AND NORMAN A. BAILY. See abstract in "Reports on Lalor Fellowship Research."

Distribution of P^{32} in Callinectes during the molting cycle. ELOISE KUNTZ.

The importance of phosphate in calcification processes during the molting cycle is well known but the distribution of phosphate for other metabolic changes which occur are less well understood. The relative uptake of P^{32} by different tissues was measured two hours after injection at five different periods in the molting cycle: (1) immediately after molting, (2) 24 hours later, (3) during the inter-molt period, (4) at an early pre-molt (white line stage) and (5) immediately before molting (red line stage). Each tissue showed a different pattern of relative uptake of P^{32}.

The shell had a high content of P^{32} only at stage (2). Concentration occurred in the hypodermis in stages 2, 3, and 4, being greatest at stage 3. The gills concentrated P^{32} during stages 1, 3, and 4. The radioactivity of the hepatopancreas was low in stages 1 and 2, then increased during stages 3 and 4 with a very high content at stage 5.

The amount of P^{32} in the green gland varied widely between individual animals but was greatest during stage 5. The extremely high content of the green gland probably was the result of the re-absorption processes since no P^{32} was detected in the sea water in which the animals were kept.

There was a progressive increase of P^{32} in the heart from stage 1–5. Very little was found in striated muscle at any stage. The ventral nerve ring concentrated P^{32} at all stages with the greatest radioactivity occurring at stage 3.

The case against a correlation between flicker and metabolism of red cells. A. K. PARPART, J. F. HOFFMAN AND J. W. GREEN.

Pulvertaft (1949) and Blowers, Clarkson and Maizels (1951) have observed that normal red cells when viewed by phase contrast microscopy show an active, continuous flicker-like alteration of optical density. They further claim that this "flicker" phenomenon disappears in the presence of metabolic inhibitors such as formalin (0.002%) and NaF (0.10 *M*). They concluded that there is a correlation between flicker and metabolism.

Normal mammalian and fish red cells examined by television microscopy in light between 3800 and 4500 Å show active flicker. Our observations lead us to believe, however, that the flicker is due to the active Brownian motion of the red cells. We have observed flicker in cells exposed to 1% formalin and 0.2 *M* NaF, both made up in isotonic NaCl. In most cases these agents cause the red cells to assume a more pronounced biconcave disc shape, and under these conditions the flicker is confined to the edge of the rounded portion of the cell. In fact, flicker is highly dependent on the shape of the red cell. Red cells in the form of flat discs (in Hayem's solution) show flicker at the outermost edge only Increasing the viscosity of the suspension medium reduces the amount of flicker. Flicker is also present in cells that have been hemolyzed by a one-quarter dilution with water and then brought back to isotonic concentration. These and other observations force the conclusion that flicker is an optical phenomenon unrelated to the metabolic activities of the red cell.

The action of crystalline ribonuclease on phosphate labeled nucleic acid; a new assay method for ribonuclease. JAY S. ROTH.

Phosphate labeled nucleic acid was prepared by growing yeast in the presence of P^{32} and isolating the nucleic acid by the method of DiCarlo. Several preparations having activities of 20,000–30,000 counts/min/mg. were used. The reaction rate was followed by the turbidimetric method of McCarty, and also simultaneous determination of the radioactive phosphate in the filtrate after removal of the precipitated acid-insoluble portion of the nucleic acid. Using a system containing 10 ml. of 0.1 *M* veronal buffer pH 7.6, 10 ml. of 0.2% labeled nucleic acid and 5 ml. of enzyme solution containing 1.25 γ of ribonuclease, it was found that the decrease in optical density of the solution and increase in radioactivity in the filtrate were both linear for the first 10 min. and then fell off rapidly. The relation between radioactive phosphate split off and enzyme concentration was also studied by incubation of 1 ml. of varying concentrations of enzyme with 1 ml. of buffer and 1 ml. of substrate for 30 min. at 25° C. This relationship was found to be linear for enzyme concentrations between 1×10^{-4} γ/ml. and 5×10^{-3} γ/ml. By using longer incubation times or substrates with higher specific activity it may be possible to increase the sensitivity considerably.

Ribonuclease may be conveniently assayed by this technique which has the following advantages: Increased specificity for the reaction, phosphate which may be split from cellular DNA by DNA ases, or from phosphoprotein or phospholipid does not interfere. Turbid or colored solutions may be used and fewer controls are needed. No digestions or colorimetric determinations are required. Using this method, the presence of ribonuclease has been demonstrated in homogenates of Arbacia eggs, sperm and *Tetrahymena gelcii* and also in intact cultures of Tetrahymena.

Changes in light emerging from the squid giant axone during activity: use of an intra-axonal light source.[1] SIDNEY SOLOMON AND JULIAN M. TOBIAS.

Toward the end of detecting, measuring and analyzing changes in protoplasmic ultrastructure occurring as a function of activity in nerve, light scattering techniques have begun to be employed. As a consequence, changes in light scattering have been observed to accompany impulse propagation. Partially to eliminate large sheath scattering and reflectivity parameters, a technique has been devised which minimizes these components and which should make the actual cellular changes more readily observable. A small quartz fiber, with white light impingent upon its protruding end, is introduced into the axoplasm of the squid giant axone, and, in this fashion, serves, at its impaling tip, to provide an intracellular light source. The light emerging from the axone at a right angle to its long axis has then been measured, using the optics of a standard microscope topped by a photomultiplier tube leading to a microammeter.

In 13 nerves, 85 periods of stimulation (175 per sec., 0.25–0.75 v., approx. 0.2 ms. pulses, duration 80–150 secs.) caused a decrease in emergent light in 61, no change in 23 and a small rise in 1. Precise time characteristics are not yet readily determinable. An initial increase in scattered light, as is often seen using whole nerve (see abstract by Bryant and Tobias, p. 205), has not been apparent in these experiments. The average maximum decrease in emergent light is about 3.3%, occurring after about 100 secs. of stimulation. Naively dividing by impulse number, this is about 0.0002% per impulse. Sub-threshold stimulation (17 trials) does not produce the change, nor is it seen with supra-maximal stimulation if the axone has failed to conduct impulses.

Light emergent directly over the needle tip has been observed, as well as the forward and back scattered light. The changes in these different sources seem to be not greatly different, one from the other, during activity. The data so far suggest that there may be a correlation between the amount of change and the initial axone diameter.

A physical study of the protoplasmic cortex of the Chaetopterus egg.[2] WALTER L. WILSON AND L. V. HEILBRUNN.

Many living cells consist of a mass of fluid protoplasm surrounded by a stiff layer just beneath the plasma membrane. This constitutes the cell cortex. In spite of the fact that the protoplasmic cortex is undoubtedly a most important part of the cell, little is known about its physical properties and how these may change during the life of the cell. The unfertilized egg of *Chaetopterus pergamentaceus* is excellent material for studying the state of gelation of the cortex. Imbedded in the cortex of this egg are numerous granules and these granules can be dislodged from the cortex by moderate centrifugal force. Following exposure to various agents, eggs are subjected to centrifugation and are then observed with high power magnification to determine whether or not the cortical granules have been dislodged. Eggs were treated with the following agents: potassium oxalate, potassium chloride, calcium chloride, acid sea water, sea water made basic with sodium hydroxide or ammonium hydroxide, ether, amyl alcohol, butyl alcohol, cold, heat, and ultraviolet radiation. Liquefaction of the cortex occurred following treatment with each of these agents except calcium chloride. Calcium produces a stiffening of the protoplasmic cortex. These results are similar to those obtained in earlier studies on the cortex of *Amoeba proteus*, thus indicating that the cortex of very different types of cells may be markedly similar in its physical properties.

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[1] This work has been aided in part by a grant from the United States Public Health Service, Panel on Morphology and Genetics, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

PAPERS READ BY TITLE

The role of the nucleolus in spindle formation. ROBERT DAY ALLEN.[1]

The germinal vesicle nuclei of the following marine eggs were studied cytologically: *Arbacia punctulata, Chaetopterus pergamentaceus, Spisula (Mactra) solidissima, Asterias forbesi* and *Nereis limbata.* Whole mounts of fixed material viewed with a phase contrast microscope revealed a double nucleolus (amphinucleolus of Wilson) as a common feature of all the eggs studied. This nucleolus consists in all cases of two parts: a bright nucleolus and one or more dark (usually smaller) nucleolini. The breakdown of the germinal vesicles was followed in all the above mentioned forms except Arbacia, where it occurs in the ovary. In all cases, the nucleolinus began to break up before rupture of the nuclear membrane and contributed to an increase in nuclear basophilia at that time. The nucleolinus or fragments thereof remained intact after rupture of the germinal vesicle and came to lie close beside the spindle area, which was surrounded by a field of basophilic granules from the germinal vesicle. Spindle fiber development is coincident with the final disappearance of the nucleolinus very close to the periphery of the spindle. Accounts of "nucleolar fragmentation" and of such fragments remaining in the spindle region are to be found in the older literature (Mead, Morgan, Tennant and Hogue, Scott, Lillie, Beams, etc.).

Indications of mucopolysaccharides have been reported in the mitotic spindle (Monné and Slautterbach) and in the nucleolus of Nereis (Kelly, using toluidine blue metachromasia). Accordingly, it was of interest to follow spindle formation with the reaction of Monné and Slautterbach. In Spisula eggs, the colors obtained were sufficiently strong to confirm the exchange of mucopolysaccharides between the disappearing nucleolinus and the forming spindle. The other eggs studied were too large to permit a clear staining reaction in whole mounts, although some eggs showed indication of mucopolysaccharide transfer. The results will later be tested on sectioned material.

Antimitotic substances secreted from eggs. ROBERT DAY ALLEN.[1]

Spisula (Mactra) solidissima eggs require several washings before they will fertilize and develop synchronously. Cleavage is delayed if thoroughly washed eggs are moderately crowded. Other marine eggs show this to a lesser degree (Glaser, Peebles, Lindahl). Spisula eggs constantly secrete a fertilization- and cleavage-inhibiting substance. Simple tests showed that this is not carbon dioxide or some other simple metabolic product. Since Spisula egg water inhibits fertilization and also agglutinates sperm, it evidently contains the "Mactra fertilizin" described by Metz. This substance is believed to be the active principle.

Fertilizin is evidently the egg jelly substance (Runnström, Tyler) which dissolves gradually in sea-water or rapidly in acid sea-water. As the clam produces large amounts of a very potent fertilizin on standing in normal sea-water, the preparations used here were supernatants from egg suspensions. Egg jelly contains a mucopolysaccharide and has the same anticoagulant properties as heparin; the same agents, including periodate, destroy these properties in both substances (Immers and Vasseur). In the present studies, Spisula egg water lost its anti-mitotic effect through treatment with 0.001 N periodate and subsequent dialysis. In order to test for mucopolysaccharides, the metachromatic reaction with toluidine blue was used; a strong positive correlation was found between metachromasia and anti-mitotic activity of egg water preparations (method of Wiame).

Spisula and Arbacia egg waters non-specifically inhibit the development of eggs of either species. These egg waters also have a similar but much less pronounced effect on Chaetopterus eggs, in which the secretion of a fertilizin has not been demonstrated. The present study suggests that the active anti-mitotic agent is identical with the sperm-agglutinating factor of egg water. However, in view of the non-specific character of the anti-mitotic effect, the two phenomena must depend upon different properties of this mucopolysaccharide. It is believed that the latter effect is due to its heparin-like composition.

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[1] Fellow, National Cancer Institute.

Birefringence of granules in the epidermis of the leaves of Spinacia oleracea L.

Ruth P. Alscher and George I. Lavin.

Detailed morphological studies of the cells of the leaves of *Spinacia oleracea* L. were made using smears of ground fresh and frozen spinach leaves and preparations of epidermal tissue stripped from living leaves and washed with 0.51% isotonic sodium chloride solution. Frozen section preparations of fresh leaves and those fixed in 4% formalin were also observed.

It was found that the leaves have a cellular structure considered typical of a mesophytic dicotyledonous leaf.

However, it was observed that pigment granules are scattered at random throughout the cytoplasm of the cells of both the upper and lower epidermis. These granules are of two distinct types: light green (an average of 1.1 μ in diameter) and yellow green (an average of 1.2 μ in diameter). When viewed under polarized light, they are birefringent and light yellow in color.

Photomicrographs of surface and cross-section views of epidermal cells under polarized light indicate that the granules are composed of two distinct regions: a centrally located, birefringent region, which may be chlorophyll and lipid in nature; and a non-birefringent region, which may be protein in nature, located peripherally. This indicates that the granules are distinct morphological units and not mere droplets of pigment, and that they are morphologically different from starch grains and crystals. A polaroid polarizer and analyzer were employed. Kodak M plates were used; these were processed in D 19 developer.

These granules bear a striking resemblance, both in size, appearance, structure and birefringence, to those which Granick and Porter observed to be embedded in the protein matrix of chloroplasts of the mesophyll cells of spinach leaves. Therefore, these epidermal granules may be pro-chloroplasts, *i.e.*, the forerunners of chloroplasts, or they may be poorly developed chloroplasts.

Interaction of sodium and potassium ions with hemoglobin and with hemerythrin.

Edwin H. Battley and Irving M. Klotz.

The selective accumulation of sodium or potassium ions within erythrocytes has frequently been attributed to selective binding by the protein which constitutes the major component of the cytoplasm. Therefore the binding of alkali metal ions by crystalline hemoglobin and by crystalline hemerythrin was examined.

Crystalline horse hemoglobin (the authors are indebted to the Mass. State Antitoxin Laboratory for a gift of horse blood) was prepared by standard procedures except that the ghosts were flocculated and precipitated by lowering the pH to 5.0–5.5. After re-crystallization the protein was dissolved in distilled water and dialyzed 2 days against distilled water at 5° C. To measure alkali metal binding, solutions of hemoglobin containing 2–12% protein were equilibrated in a cellophane bag at pH 7.2 for 18 hours at room temperature against 0.001 M NaCl and 0.001 M KCl. Separate equilibrations were carried out also in the presence of 0.1 M NH_4Cl to overcome any Donnan effect. Sodium and potassium in both the hemoglobin solution and the dialysate were measured with the Beckman flame photometer. No binding was observed.

Crystalline hemerythrin was obtained from the coelomic fluid of *Phascolosoma gouldii* by alcohol fractionation of the hemolysate at 5° C. Aqueous solutions of hemerythrin, containing 0.663% protein and which were 0.01 M with respect to NaCl and to KCl, were centrifuged at 144,000 g. Binding should result in the presence of more sodium (or potassium) in the concentrated protein at the bottom of the centrifuge tube than in the supernatant. Analyses by flame photometry gave no evidence of binding. Analyses for sodium and potassium of whole, packed cells showed that Phascolosoma corpuscles concentrate potassium from the coelomic fluid. The values were 1.85×10^{-4} M K^+ and 3.41×10^{-4} M Na^+ per gram dry weight of cells, as compared to 0.38×10^{-4} and 3.8×10^{-4}, respectively, reported in the literature for body fluid.

Chromatographic analysis of fertilizin from the sand dollar, Echinarachnius parma.

David W. Bishop.

By means of the filter-paper partition chromatographic procedure amino acid and sugar assays have been made on sand dollar egg-water. This egg-water contains fertilizin capable of reversibly agglutinating sand dollar sperm. In the material investigated the titer of this agglutinating substance in the fresh egg-water varied from 1/8 to 1/32. Essentially the same chromatograph pattern was obtained when eggs were allowed to stand in filtered sea water as when extracted with acid sea water, according to the usual procedures.

Amino acids were determined in dialyzed egg water, hydrolyzed 15–20 hours in 6 N HCl, and concentrated by partial evaporation. Two-dimensional ascending chromatograms were carried out on Whatman (no. 1) filter paper sheets. Solvents used were phenol-water (3 : 1 by weight) and n-butanol-glacial acetic acid-water (5 : 1 : 5 by volume). Ninhydrin was used as the developer. Ten spots have been provisionally identified by their R_F values as follows: isoleucine, leucine or norleucine, aspartic acid, and cystine; and probably methionine, tyrosine, threonine, glycine, arginine, and either lysine, serine, or glutamic acid. Unhydrolyzed material and sea water blanks gave no spots.

Sugar chromatograms were carried out on acid hydrolyzed egg-water, with n-butanol-glacial acetic acid-water (5 : 1 : 5) as the solvent, and ammoniacal silver nitrate as the developer. Each of four different hydrolyzed preparations gave a single spot, corresponding with the R_F value for levulose. In no case was fucose or galactose found, as reported by Vasseur for several European echinoderms. Unhydrolyzed preparations gave no spot.

This evidence suggests that fertilizin is a glycoprotein or a mucopolysaccharide (Tyler, 1948) and further contributes to the hypothesis of Vasseur (1949) that the specificity of fertilizin may lie in its polysaccharide constituents rather than in its amino acid content.

Certain of the hydrolysates, when neutralized and diluted with sea water, were capable of stimulating sluggish sperm into intense motility, probably attributable to the amino acids present.

The cytochrome system of starfish sperm. HANS BOREI AND CHARLES B. METZ.

On spectroscopic examination *Asterias forbesii* sperm suspensions present a perfectly normal cytochrome system. The positions of the absorption bands coincide with those of baker's yeast, but their relative strengths are different. Thus positions and relative strengths for the three cytochrome α-bands are ($Na_2S_2O_4$ added): ($a + a_3$) 605 (++), (b) 564 (+) and (c) 550 mμ (+++). This general pattern is similar to that found in sea-urchin sperms by Rothschild (1948), and reported in more detail by Keilin and Hartree (1949).

On addition of cyanide (in the presence of $Na_2S_2O_4$) the a-band is first sharpened up towards the red, indicating the formation of a non-absorbing $a_3 \cdots CN$-compound. The relative strengths of the bands under these conditions are: a (+++), b (++) and c (+++). When anaerobiosis develops, the absorption of $a_3 \cdot\cdot CN$ appears as a weak additional band around 590 mμ, or as a broadening of the a-band. If CO is bubbled through the suspension (in the presence of $Na_2S_2O_4$), a distinct band at 590 mμ can be observed, indicating an $a_3 \cdots CO$-compound. This band is easily bleached in the light beam of the spectroscope lamp (photoreversibility). Furthermore, the oxygen uptake of a sperm suspension (in an experiment of Sippel and Metz) was found to be inhibitable with cyanide. These observations establish the presence of the component a_3 (= cytochrome oxidase) and parallel those of Keilin and Hartree on heart muscle preparations.

A strong and broad pyridine hemochromogen band (++++) is obtained (after addition of pyridine + $Na_2S_2O_4$) around 555 mμ, and an additional weak band at 585 mμ.

The material was obtained from minced gonads of ripe animals, filtered through bolting cloth and diluted 1 : 3 with sea-water. An ordinary hand-spectroscope, illuminated with a tungsten ribbon filament lamp, was used. The light path was approximately 1 cm.

Further studies on growth and the processes of encystment and excystment in Dileptus. ANNA JOSEPHINE BRIDGMAN.

Animals of the genus *Dileptus* were excysted in the summer of 1950 and again in the summer of 1951 from soil collected in Waynesboro, Virginia. Tap or distilled water poured on the soil (which contains horse manure) will teem with these forms in a day or two.

It has been found possible to culture the animals in a balanced salt solution modified slightly from Loefer and brought to pH 8.0 by the addition of a phosphate buffer, when *Halteria* are added in some quantities as food. The *Halteria* are also grown in the balanced salt solution with added loops of *Aerobacter*.

The average size of the animals in cultures where they are multiplying is from 250–300 μ. The proboscis accounts for nearly half this length. There is a dorsal row of contractile vacuoles extending into the proboscis, and a spine at the end of the tapered trunk. The nucleus is long and sausage shaped. These characteristics are somewhat intermediate between those described by Kahl for *D. cygnus* and *D. gigas*.

A study of the encystment habits of these animals has so far revealed that when confronted with a diminishing food supply they may encyst or they may gradually decrease in size, perhaps occasionally dividing, until they are half or less of their original size. These small forms eventually encyst or die. This is in marked contrast to the situation in *D. binucleatus* where the tendency to encyst affects all the animals in a culture at approximately the same time, shortly after the depletion of the food supply. Perhaps as a corollary of this the cysts, some large and some small, show a much lower percentage of excystment with distilled water.

Oxidation-reduction dyes and "lithium larvae." MATILDA M. BROOKS.

In continuing the experiments on growth and development of marine eggs as affected by redox dyes of known electrode potentials, it was found that certain dyes initiated cleavage in unfertilized eggs of Arbacia, but that this process stopped at different stages and in most cases at the blastula stage. It was thought of interest to see wheher fertilized eggs would develop beyond this stage in the presence of these dyes.

It was found that two low potential dyes, indigo tetra sulphonate and indigo monosulphonate in concentrations from .01% to .0005% in sea water produced blastulae resembling the "lithium larvae" (extraverted gut and dumb-bell shapes). Most of them did not grow beyond this stage when kept in dye solution during two weeks of observation. A few developed abnormal plutei when transferred to sea water. The granules and gut were stained blue (indicating oxidized dye) and the cytoplasm was colorless (indicating either no accumulation or reduction).

Correlation of these results with reagents producing similar results as found by others is as follows:

Lithium reduces the rate of respiration of sea urchin eggs; CO and KCN stop oxidations; neutral red is a redox dye lower in the scale than the sulphonates; NaOH lowers the potential. One can conclude that the oxidative enzyme system is affected by all these reagents by lowering the redox potential which regulates metabolism. This permits the over-development of the micromeres at the vegetal pole of the egg, from which mesenchyme cells form. According to Child the vegetal pole has a lower metabolic gradient than the animal pole. Therefore these low potential dyes would stimulate the development at the vegetal pole and retard the development at the animal pole.

Several of these dyes at higher potentials, such as Guaiacol indophenol and methylene blue have not given such results in preliminary experiments.

A higher potential than that at the blastula stage is indicated for further development of the larva.

Hormones controlling the distal retinal pigment of Palaemonetes vulgaris. FRANK A. BROWN, JR., MILTON FINGERMAN AND MARGARET N. HINES.

Removal of one eyestalk from normal animals maintained on a white background was followed by a decrease in the degree of light adaptation of their distal retinal pigment to a significantly lower level within 15 minutes. Furthermore, dark-adapted one-eyed animals light-adapt at a significantly lower rate than normal and arrive at a final state of light adaptation comparable to that following removal of one eye as described above. These results clearly indicate that both eyestalks contribute to the normal control of the distal retinal pigment of both eyes. That the distal pigment is controlled, at least in part, by a light-adapting hormone is demonstrated by the fact that prawns dark-adapted and in darkness undergo extensive light adaptation in response to a one-minute exposure to bright light, the light adaptation becoming maximal in 30 to 60 minutes. A comparison of dark adaptation in one- and two-eyed prawns showed the rates and degrees to be indistinguishable. However, evidence for the presence of a dark-adapting hormone was obtained from two types of observations: 1) Widely different rates of dark-adaptation under different conditions, 2) dark-adapting influence of extracts of eyestalks and central nervous organs. The extent of light adaptation and the rate of re-dark-adaptation in normal prawns in darkness following a one-minute light flash could be greatly increased by simultaneously injecting extracts of eyestalks of central nervous organs. This indicated that these organs contained sources of both a light-adapting and a dark-adapting principle. There is some evidence that the tritocerebral commissure contains the dark-adapting principle in virtual absence of the light-adapting one.

Failure to obtain "cytofertilizin" from Arbacia eggs. Helen L. Byers.

Most recent workers agree that the specific sperm agglutinating agent, fertilizin, is obtained only from the jelly surrounding Echinoid eggs. However, Motomura has recently presented evidence to show that the eggs of *Strongylocentrotus pulcherrimus* contain and can secrete another sperm agglutinating agent, "cytofertilizin," in addition to the extracellular fertilizin of the jelly. Since the relation of fertilizin to the egg architecture may have an important bearing upon the role of fertilizin in fertilization, Motomura's claim is of considerable interest. Therefore, an attempt was made to demonstrate the production of cytofertilizin by Arbacia eggs. Motomura's two principle experiments were repeated. Thus, samples of unfertilized Arbacia eggs were washed in acid sea water (pH 4.2) until the egg jellies were removed and the washes no longer agglutinated sperm. The eggs were then allowed to stand in sea water for 20 hours. The supernatants from such standing eggs (2 experiments) failed to agglutinate sperm. In a second series (3 experiments) unfertilized eggs were washed (acid sea water) free or nearly free of jellies, rinsed in sea water, fertilized and shaken to remove fertilization membranes (successful for 25–50% of the eggs). In one experiment the supernatant from the shaken eggs failed to agglutinate sperm. In two experiments the supernatants from the fertilized, shaken eggs contained sperm agglutinins but not in significantly higher titer than the supernatants from unfertilized control suspensions.

In view of these results it appears unlikely that ripe unfertilized or fertilized Arbacia eggs actively secrete a sperm agglutinating agent. Evidently fertilizin is obtainable only from the extracellular jelly of these eggs.

The mesoderm question in lobeless Ilyanassa eggs. Anthony C. Clement.

In 1896 H. E. Crampton discovered that removal of the polar lobe from eggs of *Ilyanassa obsoleta* at the trefoil stage of the first cleavage results in the absence of a typical "mesoblast pole-cell," 4d, and later of the mesoblast bands. From this he was led to infer that the lobeless embryo consists only of ectoderm and endoderm. Subsequently E. B. Wilson ('04), in a study of the development of lobeless eggs of Dentalium, failed to find evidence of mesoderm in the partial larvae. It has been generally inferred, from the work cited, that removal of the polar lobe in these molluscs results in absence of mesoderm in later development.

I have removed the polar lobe at the trefoil stage in a large number of eggs of Ilyanassa and followed both the cleavage and later development. The characteristic mesentoblast cell 4d is absent, as Crampton reported. Despite this, the partial embryos and larvae arising from the lobeless eggs contain cells with the characteristics of mesenchyme, and they also develop an abundance of functional muscle tissue.

Two sources of mesoderm have been described in the classical studies of molluscan and annelidan cell lineage: 1) the primary mesoblast, from 4d; and 2) the larval mesoblast, from certain cells of the second and third quartets. In all probability the mesenchyme-like cells and muscle of the lobeless Ilyanassa larva are derived from the larval mesoblast.

Modification of the cleavage pattern in Ilyanassa through removal of the polar lobe. Anthony C. Clement.

The highly complex and precise cleavage pattern of moluscs has long been recognized as a manifestation of precocious segregation in these mosaic eggs. In a careful comparison of the cleavage in normal Ilyanassa eggs with that in eggs from which the polar lobe had been removed at the first cleavage, I have found that the lobe is responsible for certain features of the cleavage pattern in at least three of the four quartets of micromeres.

In the normal egg the D quadrant micromeres are distinctive in several respects. For example, whereas the first quartet micromeres are similar in the A, B and C quadrants and their descendants show a similar pattern of subdivision, the D quadrant first micromere is smaller, it divides later and certain of its descendants divide at a slower pace and in a distinctive pattern. Peculiarities also distinguish the D quadrant micromeres of the second and fourth quartets.

Following removal of the polar lobe—a purely cytoplasmic structure—the D quadrant micromeres no longer possess the distinctive features mentioned; so far as they have been followed, they resemble the corresponding cells of the other quadrants and conform to the same cleavage pattern.

The type of cleavage modification impressed upon the D quadrant by the polar lobe varies from cell to cell. One cell may be made to divide earlier, another later and others remain unaffected. Similarly, one cell may be made larger, another smaller and others remain unchanged. Finally, the visible composition of the cell may or may not be altered.

At least two classes of cytoplasmic determinants may be postulated for the cleavage pattern in Ilyanassa: one, independent of the polar lobe and segregated according to the same pattern in all quadrants, is responsible for the differential cleavages within each quadrant; the other, stemming from the polar lobe, is responsible for the distinctive features superimposed upon the D quadrant.

On a dinitrocresol-resistant, phosphorylating mechanism present in cell-free homogenates of unfertilized Arbacia eggs. G. H. A. Clowes, A. K. Keltch and C. P. Walters.

Confirming and extending measurements by the techniques previously described the phosphate uptake which is coupled with oxygen consumption in cell-free homogenates of unfertilized Arbacia eggs with α-ketoglutarate or pyruvate as substrate is completely suppressed by 10^{-5} *M* dinitrocresol (DNC). With hexose diphosphate as substrate, 63 per cent of the phosphorylation is resistant to 10^{-4} *M* DNC and with pyruvate plus hexose diphosphate, 50 per cent (Table I).

Table I

Phosphorus and oxygen uptake are expressed in micromoles per 0.1 ml. original eggs in 60 minutes at 20° C.

Substrate	Conc. DNC	P uptake	O_2 uptake
	moles per l. × 10^6		
α-ketoglutarate, 0.01 *M*	0	5.2	1.6
	10	0.2	1.5
Pyruvate, 0.01 *M*	0	2.6	1.1
	10	0	0.6
Hexose diphosphate, 0.0014 *M*	0	3.5	2.0
	10	2.2	2.2
	100	2.2	
Pyruvate, 0.01 *M* + hexose diphosphate, 0.0014 *M*	0	5.3	1.9
	10	2.9	1.7
	100	2.5	1.4

Homogenates of fertilized Arbacia eggs gave qualitatively similar results. Preliminary experiments suggest that homogenates of unfertilized Mactra eggs contain both a DNC-sensitive and a DNC-resistant phosphorylating mechanism. The authors thank Dr. M. E. Krahl for advice.

Phosphorus uptake of cell-free homogenates of unfertilized Arbacia eggs under aerobic and anaerobic conditions. A. K. Keltch, M. P. Smythe and G. H. A. Clowes.

When homogenates of unfertilized Arbacia eggs are incubated with hexose diphosphate and pyruvate as substrate, the dinitrocresol (DNC)-resistant phosphorylation reported in the previous communication is apparently coupled with anaerobic disposal of hexose diphosphate, as the P uptake in a nitrogen atmosphere, with or without DNC, is approximately equal to that under aerobic conditions in 10^{-4} M DNC (Table I). This anaerobic phosphorylation is accompanied by very limited lactate production. The authors thank Dr. M. E. Krahl for advice.

Table I

P uptake, O_2 uptake, and lactate formation are expressed in micromoles per 0.1 ml. original eggs in 60 minutes at 20° C.

Conc. DNC	Aerobic			Anaerobic	
	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed
Moles per l.					
0	7.2	−0.1	2.0	2.5	0.7
0.0001	2.9	0.1	1.3	2.2	1.0

On the effects exerted by calcium chloride and by freezing and thawing on the anaerobic, dinitrocresol-resistant, phosphorylating mechanism of homogenates of Arbacia eggs. G. H. A. Clowes, A. K. Keltch and C. P. Walters.

The P uptake which is coupled with oxidation of α-ketoglutarate in homogenates of unfertilized Arbacia eggs is completely suppressed by $10^{-5} - 10^{-3}$ M dinitrocresol (DNC) and by 0.0007 – 0.006 N $CaCl_2$. The P uptake with pyruvate (0.01 M) plus hexose diphosphate (0.0014 M) as substrates is reduced only to the extent of 40–50 per cent by these agents (Table I).

Table I

P uptake, oxygen uptake, and lactate formation are expressed as micromoles per 0.1 ml. original eggs in 60 minutes at 20° C.

Preparation	Inhibitor	P uptake	Lactate formation	O_2 uptake
A. Homogenate from unfertilized Arbacia eggs	0	5.9	−0.1	2.5
	0.006 N $CaCl_2$	3.2	0.1	1.9
	0.001 M DNC	3.5	−0.1	1.8
B. Homogenate A frozen and thawed	0	3.3	−0.1	3.0
	0.006 N $CaCl_2$	2.9	−0.1	2.7
	0.001 M DNC	2.8	−0.1	2.5

After twice freezing and thawing of the homogenate, the P uptake falls from 5.9 to 3.3 micromoles and this residual P uptake is almost completely resistant to either DNC or $CaCl_2$ (Table I).

The anaerobic phosphorylating mechanism, which is resistant to DNC, to $CaCl_2$, or to freezing and thawing, appears to correspond to the water-soluble, phosphorylating mechanism obtained in the supernate after centrifuging cell-free homogenates of certain mammalian tissues at 20,000 g.; P uptake by this system is also resistant to the same agents and occurs anaerobically.

On the nature of the membranes in the egg of Saccoglossus kowalevskii: effects of certain chemical solutions. ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

The reactions of the three investing membranes of the fertilized egg of *Saccoglossus kowalevskii* to isosmotic concentrations of various salt-free and various salt-deficient solutions were studied. Repeated washings in the desired solutions were used to eliminate the original sea water environment.

The outer membrane (I) is unaffected by immersion in citrated calcium-free sea water but becomes hard to see and possibly undergoes actual swelling in a salt-free solution such as urea. The second membrane (II) remains intact in calcium-free sea water but, if treated during the period of membrane elevation, achieves its full expansion more rapidly than in the normal environment. Its reaction to urea is somewhat similar though much exaggerated. The third membrane (III), which is composed of large variously-shaped translucent granules in contrast to the transparent homogeneity of membranes I and II, disappears when immersed in any of the following solutions: calcium-free sea water, sodium chloride, potassium chloride, urea, sucrose. All other granules in the perivitelline space likewise disappear in these solutions. Upon return to sea water, membrane III does not reappear as an entity. However, numerous minute translucent granules do appear, and show Brownian movement. Some of these become aligned in the position formerly occupied by membrane III, some distance inward from the visible inner boundary of membrane II. The line is very delicate and does not resemble the original membrane III.

Changes of shape and position of the egg with reference to membranes following fertilization in Saccoglossus kowalevskii. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

For about 10 minutes after their initial elevation during the fertilization reaction, the two outer membranes of the egg of *Saccoglossus kowalevskii* move away approximately equidistantly from all parts of the egg surface, simply expanding to a larger scale whatever irregular-oval shape the egg happens to have after having been shed. Then, as elevation continues, they gradually assume their "permanent" more or less spherical outline. The egg, too, becomes practically spherical during the post-fertilization period, and comes to lie near the center of the space enclosed by the membranes. Before this occurs, however, the egg undergoes a series of characteristic and orderly changes of shape and location (correlated chronologically with the separation of the first and second polar bodies). For a time it takes a very eccentric position, with the vegetal hemisphere lying very close to the membranes. In fact, there is an actual decrease in distance between the vegetal hemisphere and its surrounding membranes even though at this time the total area enclosed by the membranes is still increasing. The spherical shape and more nearly central position are finally attained before the first cleavage begins.

The occurrence of twinning in Fundulus heteroclitus after centrifugation. PAUL FORSTHOEFEL, S. J.

As part of the experimental work during the Teleost section of the Embryology Course at the Marine Biological Laboratory, Summer, 1951, several batches of *Fundulus heteroclitus* eggs were centrifuged at a force of 293 × g. (ca. 1550 RPM in an Adams Junior Safety-Head Centrifuge) for thirty minutes, at different times after fertilization. The eggs remained at the interface of layers of 0.73 *M* sucrose and sea-water during the centrifugation. 100 eggs centrifuged ten minutes after fertilization resulted in one case of twinning (1.0%), in which a main axis developed essentially normally, while a short supplementary axis at the level of the first somite showed head defects; both members of the pair had beating hearts. Forty eggs centrifuged 30 minutes after fertilization resulted in one case of twinning (2.5%), in which the members were joined at the tail end, and the axes formed a 180° angle; both twins were grossly normal in appearance. Three hundred-seventy eggs centrifuged at the early polar cap stage (ca. 30 mins. after fertilization) produced one case of twinning (0.27%), in which a small axis projected at a 90° angle at the first somite level of a larger but poorly differentiated axis; both embryos had small beating hearts in separate pericardial cavities. Of the 510 eggs centrifuged 10 or 30 minutes after fertilization, 3 cases of twinning (0.58%) resulted. Three

[illegible]

Effects of x-rays and [illegible] on the metachromasy of [illegible]. John W. Kelly

Using a spectrophotometric method, [illegible]

[illegible]

[illegible]

When heparin and protamine are combined together [illegible]

Ionizing radiation is known to increase the clotting time of blood, presumably by the release of a heparin-like anti-coagulant into the blood. [illegible]

Metachromasy associated with various cells and organs. John W. Kelly

A survey was made of metachromasy [illegible] species of animals. Toluidine blue was used as a stain [illegible]

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[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service [illegible]

The metachromasy observed is an indication of the presence of mucopolysaccharides (heparin, chondroitin-sulfuric acid, hyaluronic acid). Ovarian metachromasy in the forms studied is probably referable to a substance like hyaluronic acid, which is widely distributed in connective tissues. Metachromasy of the jelly, and possibly of the cortex, may be due to heparin-like substances. At any rate Immers and Vasseur have extracted from the jelly of a sea-urchin an anti-coagulant similar to heparin. Cytoplasmic and nuclear metachromasy cannot be so easily related to known cellular constituents.

The effect of forced aeration on the longevity of Laminaria. John W. King.

The difficulty of keeping Laminaria and other large marine algae alive in the laboratory is well known among phycologists. The writer felt that this difficulty could be overcome if the natural environment of the plants could be more satisfactorily duplicated in the laboratory.

Laminaria is usually found attached to rocks in areas where the water is in rapid motion. As the result of this rapid motion, large amounts of gases are collected from the atmosphere. With the natural environment of the plant in mind, a 1/16th inch hole was drilled in the nozzle of a salt water faucet. This hole was drilled in a downward direction at an angle of about 22.5 degrees. To the faucet was attached a rubber tube which extended to the bottom of a 4000 ml. beaker. The holdfasts of recently collected Laminaria were loosely but securely tied to rocks and placed in the beaker. The water was then allowed to run at full force throughout the experiment. Air was drawn in with a much greater force through the hole in nozzle than through a hole in rubber tube; therefore the hole in nozzle gave more desired results.

During the summer of 1950, *Laminaria agardhii* Kjellman was kept alive under the previously described conditions for a period of ten weeks with a minimum amount of sloughing off. A small amount of sloughing off could be expected since this normally takes place under natural environmental conditions as the plant grows older. At the end of ten weeks these plants were dried and they are now in the herbarium of the writer. This experiment was repeated for six weeks during the summer of 1951 and the results closely approximated those of 1950.

The skin as an organ of excretion in squids. Bruno Kisch.

The experiments were performed on squids in the first days after hatching.

If small amounts of neutral red (about 0.01 to 0.005%) are added to the sea water, the little squids are intensely accumulating the color in their bodies and already a few minutes afterwards, colored droplets appear on the surface of the skin, usually first in the apical aboral part of the body. These droplets are growing and finally discharged. They fall down to the bottom of the container. The neutral red concentrated in these droplets is now fixed, probably combined with protein or mucous substances. It is heavier than sea water and no more soluble in sea water, as the added neutral red was.

This experiment can be repeated very easily for demonstration purposes, wherever newly hatched squids are available.

A cytological study of heat-activated Nereis eggs. Roberta Lovelace.

Uninseminated eggs were activated by placing them in sea water at 35° C. for 1–5 minutes, or at 32–35° C. for 30–35 minutes. A cytological study of eggs fixed at various intervals after the termination of treatment shows that activated eggs receiving the shorter treatment (9 lots were studied) form both polar bodies, but neither nuclear nor normal cytoplasmic division occurs, and the chromosomes do not duplicate themselves. Visible components become segregated in these eggs; abnormal division into an oil-containing vegetal half and a granule-containing animal half sometimes takes place, and cilia develop on many of the eggs. Eggs receiving the longer treatment (16 lots were studied) may form both polar bodies, one polar body, or no polar body; the latter is of more frequent occurrence in all lots of eggs studied. Normal cleavages were achieved by some of the eggs in 50% of the treated lots, and a few normal trochophores developed in some of the lots with normally cleaving eggs. A cytological study of eggs from lots which gave rise to a few normal trochophores shows that eggs in normal two- and four-cell stages formed both polar bodies and appear to be haploid. Eggs which formed no polar bodies did not undergo cytoplasmic segregation; they frequently divided abnormally and many developed cilia. These uncleaved "swimmers" contain a large mass of chromatin in the animal half of the cytoplasm. This suggests that chromosome duplication has occurred, but nuclear and cytoplasmic divisions have failed.

Some cytological effects of heat on Nereis eggs. ROBERTA LOVELACE.

Inseminated eggs of *Nereis limbata* were subjected to 35° C. for 10 minutes during two critical periods of early development: (1) During the first maturation division (25–30 minutes after insemination), and (2) during the first cleavage division (70–80 minutes after insemination). A study of eggs fixed at the beginning of treatment and at intervals thereafter indicates that 35° C. interrupts the first maturation metaphase in such a way as to produce stickiness of the tetrads and irregular scattering of the chromosomal clumps in the two asters. Subsequently these eggs may give rise to one large polar body containing a variable number of chromosomes, but frequently no polar body is formed. Lagging chromosomes are frequent at the first and second cleavage anaphases, and blastomeres with heteroploid chromosome numbers may result. Many abnormal multicellular larvae develop from these eggs, but some apparently normal trochophores are produced.

When treated during late prophase, metaphase or early anaphase of the first cleavage, nuclear division proceeds but cytokinesis is blocked and binucleate cells result. If the eggs are in late anaphase or telophase when treated, cleavage proceeds normally but is accelerated. In dividing binucleate cells, each astral center divides and primary spindles are established between daughter asters. Secondary spindles develop between adjacent asters and tetrapolar figures with four secondary spindles are frequent during anaphase. Cleavage of the binucleate cells is at right angles to the primary spindles, and the egg is divided into two equal binucleate cells. In successive cleavages, the two nuclei of a cell may fuse and tetrapolar figures with the tetraploid number of chromosomes arise. Most of the larvae developing from these eggs have abnormal pigmentation and abnormal oil distribution, but some normal trochophores were present in all cultures.

Glutathione in the body fluids of marine worms. SR. ELIZABETH SETON MACDONALD AND CHARLES G. WILBER.

An increase in reducing sugars in the body fluids of marine worms follows exposures to elevated ambient temperatures. It has been suggested that part of the increase may result from increased glutathione. Worms (*Phascolosoma gouldii* and *Amphitrite ornata*) were exposed to elevated temperatures in aerated sea water and the body fluids were then analyzed for amount of glutathione.

The mean values for the control worms in mg. reduced glutathione per 100 cc. body fluid are: *Phascolosoma*, 2.5; *Amphitrite*, 23.4. After exposure to elevated temperatures the mean values for *Phascolosoma* are in mg. per 100 cc.: 32° C. for 6 hours, 5.0; 35° C. for 1 hour, 5.9; 32° C. for 1 hour, 3.4, for ½ hour, 3.5. If the worms are kept at elevated temperatures for an hour and then returned to 22° C. for an hour of recovery, the elevated glutathione values persist.

Specimens of *Amphitrite* exposed to 32° C. for ½, 1 and 6 hours, respectively, give mean values in mg. per 100 cc. as follows: 20.2, 20.0, 20.9; if 1 hour recovery follows the 6 hour exposure, the mean value is 32.8. Exposures to 35° C. give the following results: ½ hour, 18.9; 1 hour, 27.6; 1 hour followed by 1 hour recovery, 24.7.

The results to date indicate that there is an increase in body fluid glutathione in *Phascolosoma* following exposure to high temperatures, but that no such increase is found in *Amphitrite*. In the latter there is even the suggestion of a decrease under these condtions.

There is a difference in the order of magnitude of glutathione values in the two forms: *Phascolosoma* controls are less than 5, *Amphitrite* are more than 20.

In the controls for *Phascolosoma*, there is a total of 12.7 mg. per 100 cc. of glutathione; of this, 10.2 mg. is oxidized. Reduced glutathione in this form consequently makes up about 19% of the total.

On the other hand, in *Amphitrite* controls there is a total of 27 mg. per 100 cc. of glutathione; of this, 5 mg. is in the oxidized form. Reduced glutathione in this form consequently makes up about 85% of the total.

It is evident that marked differences in glutathione exist between these two species. Whether the differences can be correlated phylogenetically or otherwise cannot be decided at this time.

Detergent action of sperm extract in Nereis limbata. W. J. V. OSTERHOUT.

The sperm of *Nereis limbata* (1) penetrates the tough vitelline membrane which is very resistant mechanically and does not dissolve in 5 M H_2SO_4 or in 8 M NaOH; (2) causes the extrusion of a large volume of jelly from the egg; and (3) makes the egg segment and produce swimming larvae (trochophores).

It has been found (Biol. Bull., 1950, **99**: 362) that duponol produces all of these effects. Hence it would be possible to account for the action of the sperm by assuming that it contains a detergent acting like duponol.

We can extract from the sperm a detergent acting like duponol as shown by its effect on surface tension. The sperm is shed dry and then covered with an equal volume of sea water and heated for a few minutes at about 50 to 75° C. A stream of air is then forced through the solution which produces a mass of bubbles resembling those obtained by treating a dilute solution of duponol in the same way.

The effect is apparently not due to the body liquid surrounding the sperm since it occurs only when the sperm is killed by heat or by exposure to distilled water.

When the freshly shed unfertilized eggs are placed in such sperm extract diluted to various degrees with sea water, the jelly is slowly formed in most cases.

Behavior of jelly in eggs of Nereis limbata. W. J. V. OSTERHOUT.

When unfertilized eggs of *Nereis limbata* were transferred from sea water to distilled water external jelly was formed (E. E. Just).

Such an extrusion of jelly could be completely suppressed when the unfertilized eggs were first transferred from sea water to various solutions at low pH values for a few minutes and then placed in distilled water. Chromic acid, hydrochloric acid, picric acid, osmic acid, mercuric chloride gave this effect. Ethyl alcohol inhibited the formation of jelly but in some cases a small amount was extruded.

When the unfertilized eggs were similarly exposed to potassium chromate solution at about pH 8, the formation of jelly in distilled water was not suppressed.

The external jelly formed in distilled water could be rapidly made to contract by the following solutions at low pH values: chromic acid, hydrochloric acid, tannic acid, mercuric chloride, potassium alum, and chrome alum. The contraction by dilute hydrochloric acid was tested for reversibility. If the contraction had not progressed too far it could be partially reversed by placing the eggs in distilled water. The jelly could be made to contract for a second time by again exposing the eggs to hydrochloric acid.

The external jelly formed in distilled water could be made to dissolve by placing the eggs in a solution of potassium chromate at about pH 8.

In view of these results it may be suggested that the jelly contains some protein.

The relation of the growth of oysters to different ecological conditions in Rand's Harbor. MADELENE E. PIERCE AND JOANNE E. CURRIER.

Rand's Harbor, Megansett, Massachusetts has a peculiar gradient in physical and chemical factors due to considerable fresh water dilution at the innermost reaches of the two basins. Large natural oysters occur abundantly at limited areas of the shoreline. In 1950, two-year old oysters were placed at four stations chosen to include the widest range of environmental conditions. One station was located in the fresh water inlet of the North Basin; one directly in the small, well protected North Basin in an area of fresh-water seepage; one in the larger, wind-swept South Basin, intermediate between the fresh water inlet and the outlet to the Bay; and the fourth at the Point where conditions more nearly approximated those of Buzzards Bay.

The growing period included a total of twelve weeks, extending from mid-July to early October. Measurements of the length, width, depth, and volume of 144 oysters were made at the beginning of the experiment, at the end of the first six weeks, and at the conclusion of the twelve-week period. Growth increases in length, width, and depth were fairly constant during the entire period. The rate of volumetric increases was significantly greater during the second six-week interval. Oyster growth was most rapid in the North Basin and at the Point. Negligible growth occurred in oysters placed directly in the fresh water inlet.

A study of the chemical, physical and biological factors present in the Harbor was maintained throughout the summer. Chemical conditions and temperatures were fairly uniform and within the favorable ranges for rapid growth rates. Physical factors, such as turbidity, molar activity and exposure, varied widely and may be considered the more important limiting factors of growth. Plankton studies indicated a plentiful supply of organic food material. Pests and competing forms were definitely found to be limiting factors in certain areas, particularly at the Point. The smaller North Basin was found most suitable to oyster growth. Although the substrate was exceedingly muddy, the area was sheltered from the direct influences of the wind and turbidity was low.

This study is being extended during the summer of 1951 to a total growing season of 18 weeks. The technique has been improved and more precise data are being recorded.

Sedimentation constant of hemerythrin. ROBERT A. RESNIK AND IRVING M. KLOTZ.

Crystalline hemerythrin was obtained by alcohol fractionation of coelomic fluid from the marine worm *Phascolosoma gouldii.* Sedimentation patterns of the protein dissolved in 0.1 *M* phosphate buffer at pH 7 were photographed in the Spinco ultracentrifuge operating at 1000 revolutions per second (corresponding to about 250,000 *g*). A single symmetrical peak was observed. The following sedimentation constants, S_{20}, corrected to 20° C., were computed.

Conc. of protein, %	S_{20}, seconds
0.100	6.01×10^{-13}
0.200	6.00×10^{-13}
0.50	5.64×10^{-13}
1.00	5.34×10^{-13}

Extrapolation of the sedimentation constants to infinite dilution gives 6.2×10^{-13} sec. If the molecular weight of this protein is 66,000 (Roche and Roche, 1935), the hemerythrin molecule must have a diffusion constant near 9×10^{-7} cm.^{2}/sec. and hence must be a very symmetrical molecule, even more symmetrical than hemoglobin.

The potency of rotenone and related compounds in inhibiting division of sea-urchin eggs.[1] EDWARD F. ROGERS AND IVOR CORNMAN.

Extract of the plant Ryania contains, in addition to the muscle poison and weakly anti-mitotic ryanodine, a much more potent inhibitor of cell division. Fractionation revealed this substance to be tephrosin, a compound closely related to rotenone in chemical structure and in insecticidal action. The concentrations of rotenoids which retard cleavage of Arbacia eggs are, in mg/L: rotenone 0.002–0.004; dihydrorotenone 0.002–0.2; deguelin 0.01–0.04; tephrosin 0.02–0.1; isorotenone 0.02–0.2; rotenolone I 0.02; rotenolone II 2.0. Dehydrorotenone and isorotenolone C were without effect at 1000 mg/L (in excess of saturation). These are close to the relative toxicities for fish and insects. Dihydrorotenone, which is active against cell division over a broader range of concentrations than the other rotenoids, is more toxic to fish than to insects. This points to a different mechanism of killing (or different criteria of death) in the two phyla. Synthetic insecticides were tested in connection with the proposal of Hummer and Kenaga that rotenone and DDT act via the same pathways. DDT and methoxychlor were without effect at 2 mg/L, while dianisyl neopentane retarded division at 2 mg/L. A thousand-fold difference in permeability must be invoked if these compounds act similarly within the dividing egg. The rotenoids are similar to flavones which act as gamones for Chlamydomonas.

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[1] Aided in part by an American Cancer Society grant to the George Washington University Cancer Clinic.

Two were tested for ability to retard cleavage: isorhamnetin active at 0.2–2 mg/L and rutin, slightly inhibitory at 10–100 mg/L.

Cytochemical studies on mucous epithelial cells. R. R. RONKIN AND E. RONKIN.[1]

Since mucous cells of the hypobranchial gland of *Busycon canaliculatum* (L), a marine gastropod, are abundant and large (average length, 200 μ), they were selected for studies on mucus formation and secretion. The commonest of several types of mucous cells in the gland is club shaped; its bulbous distal portion is almost completely filled with a mucous vacuole which extends basad into the long, slender, proximal portion of the cell, almost to the basement membrane. The mucus ends close to the elongated nucleus.

The extruded mucus and the vacuolar contents usually stained uniformly and metachromatically with toluidine blue 0, indicating the probable presence of organic, polysaccharide-linked sulfate. However, vacuoles in many otherwise similar cells were stained with the "normal" color of the dye, suggesting that intravacuolar mucus synthesis was not yet completed in these cells. Alcian blue 8GS, a non-metachromatic, "mucus-specific" stain, gave results which could be interpreted similarly. Vacuolar contents showed a marked loss of stainability (Bauer-Feulgen or toluidine blue 0) following digestion with hyaluronidase. There was no observable change after tryptic digestion. The extravacuolar cytoplasm contains yellow-pigmented granules and nadi-positive granules, both of various sizes. The distribution of the nadi-positive material suggests that cytochrome oxidase is more concentrated in the distal, more bulbous portion of the cell. Lipoid granules which were stained with Sudan black B are arranged in a broad band encircling the distal portion of the mucous vacuole. Although the connective tissue of the basement membrane was found to contain glycogen (Bauer-Feulgen; controls digested with saliva), we were unable to find any glycogen in the mucous cells. Furthermore, acid hydrolysis of mucous cells scraped from the basement membrane did not release reducing sugars in detectable amounts. These observations suggest that if glycogen(or galactogen) is not present, the final stages in the formation of mucus may be coupled with aerobic utilization of lipoid material in the distal portion of the cell.

The relationship between the state of distal retinal pigment and illumination in Palaemonetes. MURIEL I. SANDEEN AND FRANK A. BROWN, JR.

The distal retinal pigment of the prawn, *Palaemonetes vulgaris*, is highly responsive to changes in amount of illumination, migrating distally in darkness and proximally in bright light. Between some still undetermined lower limit of illumination, less than 0.001 ft.c. in animals maintained in white enameled pans, and approximately 1000 ft.c. under the same background conditions, the position of the distal pigment is a function of the illumination. The study of 285 animals distributed over a series of illuminations showed the pigment 94% towards complete light adaptation at 100 ft.c., 89% at 10 ft.c., 74% at 1 ft.c., 54% at 0.1 ft.c., 38% at 0.01 ft.c. and 26% at 0.001 ft.c. Under conditions of equal illumination of the containers in which the animals were kept, animals upon a black background show greater dark adaptation of the distal pigment than those upon white. This latter difference was clearly demonstrated to be not an albedo response but rather a direct response to the total illumination of the great preponderance of the retinal elements. The black containers, which reflected about 1/70 of the light reflected from the white ones, acted precisely like a reduction of source of light to about 1/70. Therefore, unlike for the integumentary dark chromatophores, general illumination of the eye and not albedo is the exclusive light factor influencing the state of the retinal pigments. The light source itself stimulates at any given instant a very small percentage of the total ommatidial complement, and appears to have correspondingly little influence in determining the general state of the distal pigment.

Respiration of Spisula eggs. EVELYN SCLUFER.

The oxygen uptake before and after fertilization of *Spisula* (*Mactra*) *solidissima* eggs has been followed, using the Cartesian diver microrespiration technique. There seems to be no rise or drop in oxygen consumption rate following fertilization. After fertilization the oxygen consumption gradually rises in an exponential manner. At the end of this period hatching occurs, at which time the rate is 4 to 6 times the pre-fertilization rate. Later on the rate curve flattens out. The oxygen consumption per egg and hour before fertilization is ca. 2×10^{-5} μl. The diameter of the egg is 55 μ. The material is very suitable for detailed studies of the respiration during early embryonic development; the egg is fertilizable after periods up to 6 hours measurement in the diver apparatus.

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[1] This work was supported by the Office of Naval Research under a contract with the University of Delaware.

The effect of calcium and temperature on furrowing in Arbacia. Allan Scott and Richard Pollen.

If the width of the narrowing cell equator is plotted against time an S shaped curve results. Two procedures were used to obtain furrowing times: (1) precise photomicrographic measurements of the progressively changing rate; (2) visually estimating the time between the first indication of flattening and an equatorial diameter of 10 micra, closely approximating the straight line portion of the curve. Eggs were freed of their fertilization membranes. Calcium-free artificial sea water was prepared following Shapiro (1941) but adjusted to pH 8.2.

Furrowing is more rapid in Ca-free than in normal sea water. In sea water, furrowing times (method 2, 372 eggs) were 5.6, 10.2 and 20.1 minutes at 20.0°, 15.0° and 10.0° ± 0.5° C., respectively; in Ca-free sea water, furrowing (305 eggs) took 4.6, 8.1 and 14.6 minutes. Shapiro (1941, above) reported a 10% shortening of the 50% cleavage time in Ca-free sea water and implied that the developmental acceleration might be due to alterations in surface Ca-proteinates. We found a shortening of the furrowing time of 18% (20° C., method 2) in the absence of calcium but the times of 50% initiation of furrowing in Ca-free and normal sea water were within one half minute (6330 eggs). Thus we fail to confirm Shapiro in the degree of the effect.

The more rapid furrowing in Ca-free medium can be correlated with free cleavage in the absence of the hyaline layer. The simplest assumption is that the shortening of the furrowing time is primarily due to the removal of the mechanical restraint of the hyaline layer.

We have found no correlation between the rate of first cleavage furrowing in individual eggs and their rate of second cleavage furrowing, or between either of these and the time to their initiation of first cleavage.

Second cleavage furrowing averaged 3.5 minutes (Ca-free, 20° C., 97 eggs). Stratification does not alter furrowing times either in Ca-free or normal sea water. 10^{-5} *M* KCN, pH 8.2, which suppresses oxygen uptake by 50%, slowed furrowing times to 7.6 minutes (21 eggs, 20° C., normal sea water).

Preliminary measurements of birefringence changes due to electrical polarization of isolated squid giant axones. Samuel N. Shaw and Julian M. Tobias.[1]

Excitation by the action current in nerve many have as one of its components a change in protoplasmic ultrastructure (molecular-macromolecular) which might be expected to entail a change in birefringence. To date, no such change has been found to accompany propagated impulses (Schmitt and Schmitt, Jour. Physiol., **98** (1940): 26). Since polarization by non-polarizable electrodes may mimic the electrical components of the propagated disturbance, however, and, because of increased intensity and duration, may make them more readily detectable (Tobias in *Modern Trends in Physiology and Biochemistry*, Academic Press, 1951), we have measured changes in birefringence of squid giant axones as a function of such polarization.

Axones were isolated in sea water and mounted in a mineral oil-filled, plastic, glass bottomed trough on sea-water-soaked thread electrodes. The polarizing voltage (15 v.) was applied via calomel half-cells, both current and voltage being measured throughout. Retardation was measured at 1–2.5 mm. from the electrode being studied by means of a Berek compensator mounted in a Spencer, polaroid, polarizing microscope. Diameter changes were measured with an ocular, filar micrometer.

[1] This work has been aided in part by a grant from the United States Public Health Service, Panel on Genetics and Morphology, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Normal axonal birefringence, using the whole sheathed axone, was found to be positive with respect to the axonal long axis, and was about 1.64×10^{-4} mu per mu. This compares favorably with figures given by Schmitt, Bear and Young, 1.5×10^{-4}. Initial cathodal polarization produced a fall in birefringence (1.9×10^{-6} mu per mu per min., aver. of 5); initial anodal polarization produced a larger fall (4.6×10^{-6} mu per mu per min., aver. of 4). Cathodal polarization, if it followed anodal polarization, however, in all cases favored a rise in birefringence, whereas anodal polarization following cathodal polarization again produced a fall. Spontaneous changes following cessation of the polarizing current have not yet been measured, and these are required before much significance may be attributed to these apparent reversals.

Reduction of neotetrazolium chloride by X-radiated root tip cells of Allium. B. P. Sonnenblick.

Reports by several investigators (Antopol, Glaubach, Goldman, Narahara, Sonnenblick) indicate that the enzyme systems responsible for the reduction of neotetrazolium chloride (NT) are present in many living tissues. The faintly yellow salt undergoes a progressive series of color changes and is finally converted to an insoluble purple-black granular compound located characteristically in the cytoplasm about the nuclear membrane.

Studies on the mechanism of NT reduction show that active —SH groups play a significant role in the process. Since reports exist indicating that moderate amounts of X-rays effect inhibition of sulfhydryl enzymes *in vivo* and *in vitro*, it was decided to study the reduction of NT by X-radiated root tip cells. Dormant bulbs and actively growing roots were irradiated and excised roots subsequently tested at various periods following exposure. Roots were given 300, 600 or 900 roentgens, while bulbs were exposed to 300, 1200 or 2400 roentgens.

Tests indicate that cells given treatments causing a moderate frequency of chromosomal aberrations show no inhibition in the speed and intensity of NT reduction. With dosages causing severe nuclear damage the results are variable, but even after such exposures NT reduction can proceed as well as in control material. To interpret these results the following must be considered: (1) the greater sensitivity of dilute aqueous solutions of enzymes to irradiation than when accorded the protection of other substances within cells, (2) the possibility of unequal inhibition of different sulfhydryl enzymes by X-rays, and (3) the role played by other than —SH enzymes in NT reduction.

Very recently Le May of Cambridge University reported no inhibition of the —SH enzyme succinic dehydrogenase in rat kidney slices and homogenates following 400 r or 800 r of total-body X-radiation, a result *in vivo* contrasting markedly to the effect *in vitro* of X-rays on dilute solutions of that enzyme.

A blood anti-coagulant from surf clams.[1] Lyell J. Thomas, Jr.

According to Heilbrunn many of the colloidal changes taking place in cells are similar to those which occur in the blood clotting process. If this is true, then one would expect to find both clotting and anti-clotting substances in all types of living systems. For this reason I have been examining various invertebrate materials for their content of heparin-like substances. A potent blood anti-coagulant has been obtained from the common surf clam, Spisula. This anti-coagulant gives a strong metachromatic reaction with toluidine blue which is an indication that it is chemically similar to heparin.

After removal of shell, visceral mass and gonads, the clams were extracted by the method of Charles and Scott for the isolation of crude heparin. This was followed by a phenol-water partition as recommended by Homan and Lens. The product obtained in a yield of about 500 mg. per kilogram still contains colored impurities and a trace of protein material.

Anti-coagulant activity was assayed by a method similar to that of Chargaff. Untreated hen plasma diluted with test solution and thromboplastin formed the clotting system. All preparations from Spisula were the equivalent of or better than ordinary commercial preparations of heparin (Hynson, Wescott and Dunning).

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[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

The metachromatic activity of the Spisula preparations and of various commercial heparins was tested for me by Mr. John W. Kelly. In all cases, metachromatic activity paralleled anti-coagulant activity.

Further work is under way to determine the chemical nature of this substance from Spisula and to determine whether it can be obtained from other invertebrates.

Phosphorus metabolism in developing sea urchin embryos as a function of temperature. CLAUDE A. VILLEE AND DOROTHY T. BALZER.

Previous experiments had shown that low temperature markedly reduced the uptake of P^{32} by fertilized *Arbacia* eggs and it was concluded that phosphorus enters the cell by an enzyme-controlled metabolic process rather than by simple diffusion. In the present experiments, eggs were fertilized in sea water containing 3 to 5 μC of P^{32} and incubated seven hours at controlled temperatures ranging from − 12° C. to 28° C. After incubation they were centrifuged lightly to remove the sea water, homogenized in ice-cold 7% trichloracetic acid and fractionated by a modified Schmidt-Thannhauser method. The specific activities of the fractions were calculated from Fiske-SubbaRow phosphorus determinations and P^{32} analyses made with a dip counter. When the logarithm of the specific activity of the RNA P is plotted against the temperature, a straight line is obtained. Thus, the incorporation of P^{32} into RNA is described by the equation, Specific Activity $= e^{kt}$, with k having a value of 0.036. Similar plots of the other fractions did not give straight lines; the plot of the acid soluble P fraction fell below a straight line at higher temperatures and the plots of the DNA P and phospholipid P increased with temperature more than by a straight line relationship.

Studies of phosphorus metabolism in ovarian eggs of the salamander, Triturus pyrrhogaster. CLAUDE A. VILLEE AND WILLIAM R. DURYEE.[1]

Ovarian eggs were dissected free from ovarian tissue in amphibian-Ringer's solution and separated into two groups (small, 1.2 mm.^{3}; large, 6.3 mm.^{3}). About 200 eggs were placed in 35 ml. of bicarbonate-buffered Ringer's (pH 7.6) containing 1 μc/ml. of P^{32}. After 6 or 24 hours, the eggs were removed, rinsed in Ringer's, blotted and weighed. They were homogenized in ice-cold 7% trichloracetic acid and fractionated by a modified Schmidt-Thannhauser procedure. Phosphorus determinations were made by the Fiske-SubbaRow method and P^{32} determinations with a Solomon-Estes dip counter.

Salamander eggs differ from Arbacia eggs in having a large phosphoprotein fraction; 74% in small and 90% in large eggs of the acid insoluble phosphorus is phosphoprotein phosphorus. In both sizes the RNA phosphorus turns over faster than the DNA phosphorus. In small eggs (without yolk) the phosphoprotein phosphorus turns over faster than either RNA or DNA, but in large eggs (with yolk) the phosphoprotein phosphorus turns over at a slower rate than either RNA or DNA. Both large and small eggs show similar rates of turnover in their acid soluble P fractions and in their phospholipid P fractions.

Large eggs have considerably more phospholipid P than small ones, whether calculated as mg. P per gm. wet weight of eggs or as μg P per egg. Large eggs have smaller amounts of DNA P and RNA P than small ones, calculated as mg. P per gm. eggs: namely 2.65 times as much DNA P and 2.05 times as much RNA P. Both DNA and RNA P turn over more slowly in the large eggs than in the small ones.

Feulgen stains show that the nucleus is nearly DNA-free, except for the vesicle cores of each nucleolus. Subsequently, nucleolar DNA is extruded through the nuclear membrane and disperses in vacuoles toward the cell surface.

Diurnal rhythm in the controlling mechanism of distal retinal pigment of Palaemonetes. H. MARGUERITE WEBB AND FRANK A. BROWN, JR.

Palaemonetes maintained in constant darkness exhibit a diurnal rhythm in the characteristics of their response to a one-minute flash of 250 ft.c. illumination. This takes the form of variations in the extent of light adaptation and in the rate at which the animals return to a condition of complete dark adaptation. This rate is maximal at approximately midnight, rapidly decreases to a minimum at 5 or 6 A.M., increases at first rapidly and then more gradually until 7 or 8 P.M. when there is a second rapid increase to the maximum rate at midnight. It is reasonable to assume that the variations in the extent of light adaptation and the rate of subsequent re-dark-adaptation following one-minute light flashes were due to differences in the availability of dark-adapting hormone in the 24-hour cycle since animals maintained in constant darkness show approximately equal ability to light-adapt throughout the 24-hour cycle. Animals maintained under conditions of continuous illumination of 250 ft.c. are able to dark-adapt at nearly equal rates at any time during the 24-hour cycle, indicating a ready availability of the dark-adapting hormone. It is concluded that constant illumination at 250 ft.c. suppresses the rhythm of availability of dark-adapting hormone. A persistent rhythm is directly observable in normal Palaemonetes under conditions of intermediate states of light adaptation such as obtains at 10 ft.c. on a black background. Under these latter conditions this rhythm was of significant but small amplitude involving about 25% of the normal total pigmentary range.

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[1] National Cancer Institute, U. S. Public Health Service, Federal Security Agency.

The effects of x-rays upon dividing and non-dividing cells: Paramecium caudatum. RALPH WICHTERMAN.[1]

Clonal cultures of *Paramecium caudatum* were cultivated in desiccated lettuce infusion and inoculated with *Aerobacter aerogenes* as the food source. While division may be completed one-half hour from the time a spindle-shaped dividing cell can be first recognized, the process may last 2 or more hours before final separation into two daughters. In the very late division stage in which the cell is almost completely constricted into two, the forming daughters may be held together by a fine strand of protoplasm for an hour or more before breaking. In contrast to vegetative cells, the dividing cells from heavily bacterized cultures are sticky and readily adhere to the walls of capillary pipettes. In such rich cultures, chains of 3 or 4 cells are commonly seen. Like vegetative specimens, such chains of fully formed but occasionally smaller animals swim in left spirals. Like typically dividing paramecia, the chains are more abundant at the bottom of a culture.

Many isolated late dividing and non-dividing (vegetative) stages were irradiated with 300,000 r, immediately examined and maintained in isolation culture at 24° C. Irradiation of late dividing cells greatly prolongs the division process. Such dividers commonly remain suspended in division, yet swim actively, for 6–48 hours, occasionally longer. Generally the X-rayed dividing cells do not live to produce daughters but if produced, they persist for a day or more then die. Occasionally one of the daughters produced by an irradiated divider dies while the remaining daughter lives to divide later into two. This may be repeated but specimens usually die. In exceptional instances, an irradiated divider after gradual recovery from X-ray effects may produce viable daughter cells which yield survivors that later approach the control fission-rate. Irradiation of dividers can produce giant C-shaped monsters from which normal appearing specimens may originate. While irradiated dividing cells commonly die during the process of division, when given the same X-ray dosage non-dividing cells from young cultures commonly live to produce viable progeny after overcoming an initial division lag. However, non-dividing cells from older cultures are almost instantly killed by the same X-ray dosage. Irradiated, bacterized culture fluid has no lethal effect upon paramecia.

The effects of x-rays upon conjugant and vegetative stages of Paramecium calkinsi. RALPH WICHTERMAN.[1]

Clones of brackish-water *Paramecium calkinsi* of opposite mating type have been maintained in continuous cultivation over 4 years in lettuce infusion-sea water cultures inoculated with *Aerobacter aerogenes.* When in proper nutritive condition, specimens of opposite type, when mixed, form the characteristic clumps, enter into conjugation any time of day and at 25° C. separate 12–13 hours later. Lower temperatures lengthen the process.

Irradiation of joined conjugants applied at the rate of 7420 r per minute until 250,000 r are reached prolongs the conjugation process. Most of the irradiated conjugants recover but do not begin separation into exconjugants until all of the control conjugants have separated. In a sample experiment using 108 isolated control conjugants, all separated into exconjugants within a period of 2 hours completing the process within 13 hours. On the other hand, 112 irradiated conjugants separated over a 5 hour period with the last conjugants separating approximately 22 hours after mixing the opposite types. Unirradiated vegetative and conjugant stages characteristically spiral to the right in this species. The same is true for the irradiated conjugants even in late stages where a thick strand of protoplasm is all that holds the conjugants together in the region of the paroral cones.

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[1] Aided by a grant from the Committee on Research and Publication, Temple University and performed under Contract NR 135–120, Office of Naval Research, Department of the Navy.

Recent, early stage conjugants, conjugants near the completion of the process and young exconjugants were irradiated to determine possible differences in susceptibility to the X-rays. No well-defined difference could be detected. Most of the irradiated young and old conjugants and the young exconjugants survive. However, with vegetative stages from a culture of one mating type reactive for mating, fully 90% of the irradiated specimens die after 24 hours. The survivors overcome the initial division lag—not dividing for 3 days—then the fission-rate gradually approximates that of the controls. When specimens are taken from mating-reactive cultures and irradiated, there is seen to be a difference in X-ray susceptibility in the opposite mating types. With one mating type, nearly all the vegetative stages are killed almost immediately after maximal dosages while comparatively few are killed from those of the opposite type even when both are of the same cultural age. Irradiated, bacterized culture fluid has no lethal effect upon the paramecia.

pH determination in the giant nerve fiber of the squid. Floyd J. Wiercinski.

The problem of determining the exact pH of nerve tissue presents numerous difficulties. Most important of all, most nerve tissues are opaque to light so that it is impossible to use pH indicators. The giant nerve fiber of the squid offers some possibility for obtaining some data for the determination of the pH of nerve axoplasm with indicators since the fresh tissue is transparent to light.

A squid was decapitated. The mantle tissue was immersed under mineral oil; pH determinations were attempted *in situ* by injecting indicators into the nerve with a quartz micropipette. In later experiments the giant nerve was dissected out from the mantle under oil. The sheath was removed from sections of the nerve. The nerve was then prepared in a hanging oil drop for microinjection. The water soluble indicators were used. Reference was made to micro-color standards for the pH reading. The results were recorded on Kodachrome film.

Tests with phenol red showed a pH < 7.0, brom thymol blue > 6.4–6.8, brom cresol purple > 6.0–6.8, brom cresol green > 5.6, chlor phenol red > 6.0, brom phenol blue > 4.6.

The results obtained indicate a pH somewhere in the neighborhood of neutrality for the clear axoplasm of the giant nerve fiber of the squid that is put under oil and measured immediately. The nerve sheath and stellate ganglion indicated a more alkaline pH reading. Crushing a nerve fiber not under oil showed more acid pH readings.

Activity of chromatophores in the integument of the squid. Floyd J. Wiercinski.

A squid was cut across the head immediately posterior to the eyes. This cut separates the cerebral ganglion from the pleuro-visceral ganglion. It was noted that the chromatophores expanded to a dark red color. The decerebrized squid carried on the function of locomotion for a short period of time. Ink invariably was released. Later the chromatophores contracted and the squid appeared white in color.

A squid was decapitated by a cut made across the neck posterior to the pleuro-visceral ganglion. The chromatophores immediately contracted and the squid became white. In most cases ink was not released and the squid lost all locomotion.

Pieces of integument were removed from a squid. When viewed under the compound microscope the chromatophores appeared to be inactive but in a few minutes they began to contract and expand. This phenomenon seemed comparable to tissue shock.

Some chromatophores appeared quiescent and contracted, others were active at a rate of about 8 to 12 contractions per minute. They appeared in various shades of yellow, red and black. Radiating strands of muscle fibers attached to each chromatophore were seen. When the chromatophore was expanded the muscle fibers were in contraction. These phenomena were recorded on Kodachrome film.

A number of substances were tried on the activity of chromatophores in the excised epidermis of the squid. 0.1 *N* HCl fixed the chromatophores in the contracted state and the muscle fibers in the extended state. *M/2* NH_4Cl in sea water, pH 6.55 changed the rate of contraction of a dark red chromatophore from 8 per minute to 42 per minute. A 1 : 100 heparin in sea water increased the frequency of contraction; 1 : 500 hyaluronidase had some effect in causing expansion of the chromatophores (muscle fibers contracted).

It is concluded that a chromatophore in the integument of the squid is contracted and expanded by means of muscle fibers. The cerebral and pleuro-visceral ganglion play a role in keeping the muscle fibers contracted. The chromatophores are then in the expanded state. A new approach to the study of muscle contraction may be found here.

Lipids in frog blood. CHARLES G. WILBER, JOEL R. LIEB AND G. M. SLANE.

In connection with a survey of the lipid constituents of body fluids from a wide variety of animals, a study of the lipids in the blood of various frogs was begun. Routine colorimetric procedures were done. In the species *Xenopus laevis* the mean blood fatty acids are 520 mg. per 100 cc. whole blood, with a range of 490 to 540 mg. per 100 cc. Lipid phosphorus in this form averages 20.7 mg. per 100 cc. with a range of 16 to 27 mg. per 100 cc.

In *Rana pipiens*, erratic results for total fatty acids of the blood have to date prevented any reporting of these values. Lipid phosphorus of the blood from frogs kept at 18° C. for several weeks averages 10.5 mg. per 100 cc. with a range of 9 to 12 mg. per 100 cc. In frogs kept at 27° C. for 24 hours the average blood lipid phosphorus is 8.9 mg. per 100 cc. with a range of 7.5 to 10.5 mg. per 100 cc. In frogs kept at 35° C. for 24 hours the average blood lipid phosphorus is 11.2 mg. per 100 cc. with a range of 9 to 13 mg. per 100 cc. Although the mean values suggest a change of lipid phosphorus content of frog blood with increased temperatures, the differences are not statistically real. These results are not in accord with those on the turtle in which the blood lipid phosphorus decreases with increased ambient temperatures. It is, moreover, apparent that the average value of lipid phosphorus in the blood of Xenopus is closer to that of the turtle (19 mg. per 100 cc.) than of the frog Rana. The significance of this latter fact is not clear.

Protein molecules with relatively low molecular weights? DOROTHY WRINCH.[1]

Despite the vast variety of sizes and shapes of native protein structures, evidence compatible with the idea of one or a homologous series of molecular skeletons characterizing proteins in general is accumulating. The variety would then be interpretable in terms of *particle* structures comprising a wide range of different numbers of the common skeleton or skeletons, variously substituted and variously arranged.

The insulin structure, MW ca. 36,000, is known to be a trimer of structures with estimated molecular weight 11,700 (Low, 1949): but subsequent work (Fredericq and Neurath, 1950) suggests there is a further dissociation, into entities with MW ca. 6000. Such an entity would have a *skeletal* residue number of about 48–50, following Chibnall's amino acid analysis and allowing for the *extra-skeletal* residues with free α-amino or α-carboxyl groups. This result accords well with the previously made suggestion of the possibility of 6 cages each comprising 48 skeletal residues plus extra-skeletal residues in the original insulin structure (Wrinch, 1948).

Such a type of low molecular weight entity throws light on the case of horse myoglobin, a structure with ca. 147 residues. It seems impossible to interpret the vector maps derived from this X-ray study, except on the assumption that the structure is a particle with more than 2 sub-units. A structure comprising 3 sub-units would allocate about 49 residues to each, fitting well with the proposed entity. Other proteins which may well fit into the picture of this single type of entity, in various numbers and in various arrangements, include whale myoglobin, horse hemoglobin, pepsin and β-lactoglobulin and even such relatively large proteins as edestin, tobacco seed globulin and excelsin.

Further confirmation of this viewpoint is being sought in a detailed study of the intensity maps obtained in X-ray studies of wet crystalline proteins.

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[1] This work is supported by the Office of Naval Research under contract N8onr–579 with Smith College.

Water in protein crystals. DOROTHY WRINCH.[1]

It is well known, from X-ray studies, that crystalline proteins containing prodigious complements of water yield intensities which indicate a high degree of orderliness extending down to atomic dimensions. For wet insulin, there is no less than 46 per cent water by weight, if a dry molecular weight of ca. 36,000 be assumed. For horse methemoglobin, still larger complements of water are present and crystals yielding intensities down to atomic spacings. The effect on the intensity maps of reducing the water complements shows that not only the protein structures but also some considerable proportions of the water molecules form orderly patterns at the atomic level. It follows that the role played by the water molecules in determining the intensities, and the vector maps derived from them, is likely to be of crucial importance.

Accordingly a study of the vector maps to be expected of molecular crystals containing a patterned medium has been undertaken. It proves possible to trace the accentuation of the nodes of the lattice of the medium and to determine the manner in which it depends upon the particular spaces occupied by the molecules themselves. In an application to the case of wet insulin, it is found that the well-marked peaks in the vector map at about 5A from the origin may be due in part to the existence of water molecules in clusters within the crystal.

The study is continuing.

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[1] This work is supported by the Office of Naval Research under contract N8onr-579 with Smith College.

REPORTS ON LALOR FELLOWSHIP RESEARCH

A new DNA fraction of the cell nucleus. JAY BARTON II.

The action of crystalline DNase on nuclei may be conveniently studied and quantitatively characterized by using isolated nuclei as the substrate. The following conclusions may be drawn: (1) Only a part of the DNA can be removed by the enzyme. (2) No protein is released with the DNA. (3) Calcium is the only other component of the nucleus released along with the DNA so far discovered.

The enzymatically resistant fraction cannot be removed by repeated digestion with fresh enzyme, by washing with water, or by washing with salt or buffer solutions of moderate ionic strength. The fraction can be removed as a complex of nucleic acid and protein by strong salt solution, 1 or 2 *M* NaCl. The complex removed in this way is not dissociated by saturated ammonium sulfate, retaining constant properties through repeated precipitations and washings and resolutions.

The amount of the resistant fraction in various species is as follows: Beef thymus lymphocyte nuclei, 77%; carp sperm, 82%; Asterias sperm, 74%; Pecten, 89%; Mytilus sperm, 84%; Arbacia sperm, 74%; turtle (Caretta) erythrocyte nuclei, 80%; Mactra (= Spisula) sperm, 40%. In all species except Mactra the basic protein of the nucleus is of the histone type. In Mactra the basic protein more closely resembles a protamine.

It is postulated that the resistant DNA is part of a complex of nucleic acid and protein held together by bonds other than electrostatic, probably co-valent.

Some exploratory experiments were performed testing the completeness of extraction of nucleoprotein from sperm by the classic procedure using 1 or 2 *M* NaCl. Tentatively, the results indicate that in those species having a small enzymatically resistant fraction, a small fraction resistant to NaCl extraction may also be discovered. In Mactra the indications are that there is a rather large fraction of DNA resistant to salt extraction. The identity of the latter fraction to the enzymatically resistant fraction has not yet been established.

Carbonic anhydrase and calcification in the fiddler crab (Uca). REINHOLD BENESCH AND RUTH E. BENESCH.

This work was designed to test the hypothesis that carbonic anhydrase functions in the formation of the crustacean exoskeleton. Molting was induced in the fiddler crab (Uca) by ablation of the eye stalks. Immediately upon completion of ecdysis the animals were placed in sea water containing varying concentrations of sulfanilamide, a powerful carbonic anhydrase inhibitor. After varying lengths of time treated animals and controls were dried for five hours at 90° C., weighed and ashed. Ca plus Mg was estimated by the Versenate method. The following observations were made: (1) The crabs were found to be more resistant to the drug than vertebrates, since they easily tolerated up to 50 mg.% of sulfanilamide for five days. (2) The Ca plus Mg content of 20 sulfanilamide-treated crabs was compared with that of 9 controls five days after molting. The average values were 8.3% for the treated and 10.2% for the control group. This difference is not statistically significant. There was, however, a striking inverse correlation between the size of the animal and its rate of calcification. (3) In a short term experiment, in which the animals were kept in 50 mg.% sulfanilamide for 24 hours after molting, the mean calcium content of five treated animals was 4.4% and that of three controls was 6.3%. The standard error of the difference between these means was 0.9 which makes this difference just significant. These preliminary results would indicate that carbonic anhydrase might play a part in the formation of the crustacean exoskeleton, but they require confirmation with a larger number of animals.

Studies on the nonsaponifiable fraction of lipid material from marine animals. Leon S. Ciereszko.

Except for the observation by Bergmann *et al.* that cholesterol is the principal sterol of the tunicate, *Styela plicata*, nothing appears to be known about the nonsaponifiable matter of Protochordata. To extend our information along this line, two other tunicates, *Amaroucium constellatum*, and *Molgula manhattensis*, were worked up for nonsaponifiable matter. Wet *Amaroucium* yielded 0.4–0.5% total lipid. Of this 16% was nonsaponifiable. About 25% of the nonsaponifiable matter was obtained as crude sterol. Molgula, stripped of tests, yielded 0.08% nonsaponifiable matter. The sterols of the tunicates were isolated in crude form but their identification was not completed.

It was found that the pyloric caeca of the starfish, *Asterias forbesi* are rich in lipid including phospholipid. The nonsaponifiable matter of starfish caeca is rich in glyceryl ethers (about 60%), while no glyceryl ether could be detected in the lipids of starfish skin.

Urea adduct formation was applied to the fractionation of nonsaponifiable matter. Pure glyceryl ether fractions were isolated via their urea adducts from the liver oil of the shark, *Galeocerdo arcticus*, and from the fat of pyloric caeca of Asterias.

The tunicate, Botryllus, was found to contain a water soluble pigment which gave a cherry red color with concentrated sulfuric acid.

Oils of a number of marine animals, as well as aqueous extracts of Molgula and Amaroucium were prepared for later study.

Enzymes of a ribose-forming pathway during the early embryonic development of Chaetopterus. Seymour S. Cohen.

A number of marine forms have been found to contain dehydrogenases essential to the oxidative formation of pentose phosphate from glucose-6-phosphate, *i.e.*, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The reduction of the coenzyme, TPN, occurs in each enzymatic reaction and the absorption of reduced coenzyme is used in the assay of the enzyme in homogenates.

It appeared of interest to determine the course of synthesis of these important enzymes in the early embryonic development of the polychaete worm, Chaetopterus, as well as the formation of ribose nucleic acid (RNA) in the embryo. An unfertilized egg contained about 2.6×10^{-5} micromoles of RNA-ribose and enough of each dehydrogenase to generate this amount of ribose in ca. 10 hours at 25°. In a 12 hour period at 22–23° in which the fertilized ovum segmented and differentiated to a ciliated swimming form of over 100 cells, there was no significant change in the RNA content of the embryo nor in the amounts of the two dehydrogenases.

Cations were observed to activate each enzyme. Mg^{++} and Ca^{++} were equivalent and optimal at .033 *M*, activating to twice the extent of Na^{+}, K^{+}, or Li^{+} which were optimal at 0.2 *M*. Concentrations higher than the optima were markedly inhibitory.

In the assay, embryos were homogenized at 0° in H_2O or 0.2 *M* NaCl and sedimented, the supernatant fluid containing the activities. Aliquots of 0.1 cc. were added to a mixture containing 1.0 micromoles substrate, cation at optimal concentration, and 0.025 *M* glycyl-glycine buffer at pH 7.4. The reaction was begun by the addition of 0.1 micromole of TPN in a final volume of 3.0 cc. It was found that the initial rate of reaction was proportional to enzyme concentration. The optimal pH range extended from pH 7.2 to 8.2.

Cyto-embryology in sea urchins. Katsuma Dan.

The movement of the cortex of the eggs of *Arbacia punctulata* in Ca-free medium is followed by the shift of the pigment granules embedded in the cortical layer. In a dividing cell, the cortex of the cleavage furrow acquires a deep pink color while the color on the rest of the surface fades, showing the thickening of the furrow cortex. The pigment accumulation remains for a few minutes after the cleavage, then soon dissipates, so that during the resting stage, the furrow region becomes almost colorless while the polar surface assumes a deep pink color. Before the second cleavage, the pigment becomes uniformly distributed again. Since similar movements are repeated through the next cleavage cycle, at the interkinesis of the four-cell stage the cortex moves out of the interior of the larva and comes to lie on the periphery.

In sea water, the egg surface is normally bound to the overlying hyaline layer by the attachment fibers. Consequently, the cortex always remains on the periphery of the larva. The fact that the unrestricted cortical movement in Ca-free medium can give rise to the same distribution of the cortical material as that in sea water seems to be very significant from an embryological standpoint.

The tendency for the granules to return to a uniform distribution before each division must be connected with the division activity, and this accounts for the rounding-up of the blastomeres at this period.

The behavior of the hyaline layer after the mesenchyme cells free themselves from it is followed in Arbacia and Echinarachnius.

Auxin control of rhizoid-formation in Bryopsis. W. P. JACOBS.

The experiments of 1950 which verified the hypothesis that auxin controls rhizoid-formation in Bryopsis, a coenocytic alga, were confirmed and extended. Turning Bryopsis upside down and burying the shoot-tip in sand causes the buried tips to continue their growth as rhizoids; if the light intensity is high enough, the former rhizoid-forming bases will produce positively phototropic shoots. This reversal of polarity is usually preceded by migration of chloroplasts out of the buried portion and into the lighted portion. If the stem is ligated below the region of highest concentration of chloroplasts and before this migration of chloroplasts occurs, then regeneration in the region of the ligation follows the original polarity of the plant (*i.e.*, a shoot regenerates just above the ligation, a rhizoid usually regenerates just below it). These experiments do not support the idea that migration of chloroplasts is a necessary prerequisite for reversal of polarity.

Rates of feeding and oxygen uptake in some filter feeding marine invertebrates. C. BARKER JØRGENSEN.

As part of a comparative study of feeding in filter feeders, feeding rates were measured in the oyster, *Ostrea virginica*, and two ascidians, *Ciona intestinalis* and *Molgula manhattensis*. Feeding rates were computed from the rates in which the experimental animals removed finely dispersed graphite (particle size about 1 to 4 μ) from a suspension of known volume. Graphite concentrations were measured by means of a photoelectric colorimeter. In order to make possible a comparison of feeding rates in different filter feeders, the volumes of water filtered were expressed relative to the simultaneous oxygen consumption.

The rate of feeding was approximately the same in the three species. In the oyster 12–24 liters of water were filtered for each ml. of oxygen uptake. In Ciona and Molgula the figures were 8–20 liters and 10–20 liters, respectively. These values are of the same order as filtering rates previously measured in *Mytilus edulis*, *Pecten latiauratus*, and *Ciona intestinalis* (unpublished).

The ascidians retained graphite effectively only when the gill basket was covered by a sheet of mucus, secreted by the endostyle. When such mucus was present no graphite passed the filtering organs. The smallest particles were less than 1 μ in diameter. Previously it has been found that some proteins, like hemocyanin and hemoglobin, easily pass through the mucus sheet (unpublished). In the oyster graphite particles larger than about 2–3 μ were apparently completely retained by the gills whereas smaller graphite particles were able to pass through the gill filters.

The metabolism of α-glyceryl ethers in marine animals. M. L. KARNOVSKY.

The lipids of marine animals are notable for their content of α-glyceryl ethers, but although many reports of the isolation of these substances have appeared, there is no literature on their metabolism.

A rapid, sensitive method for determining α-glyceryl ethers colorimetrically has been worked out. The method depends on the specific oxidation of the glyceryl ether with periodate, and determination of the formaldehyde generated. The lipids of several species were analyzed, and Woods Hole species were found little different from their South Atlantic counterparts.

The metabolic problem was approached from three angles:

1) A possible enzymatic hydrolytic split of the ether link, with liberation of long-chain fatty alcohols, was postulated. Such enzymes have been sought, using a variety of water-soluble ether substrates, but without success.

2) The possibility of the reversible reduction of the ester link to an ether link at the α-position of the triglyceride has been examined, by using starfish digestive glands incubated aerobically or anaerobically. Highly oxygenated conditions appear to reduce the titer of α-glycerol ethers.

3) The turnover rate of the glyceryl ether fraction could be compared with that of triglyceride and phosphatide, by the use of C^{14}-labelled glycerol. The experiments are not completed.

The possibility of the existence of a phospholipid analogous instructure to the α-glyceryl ethers has been investigated. Suggestive evidence for this has been obtained. In summary: A petroleum ether-soluble, organic phosphorus-containing substance can be isolated from the saponified phosphatide fraction of the liver lipid of the tiger shark (*Galeocerdo arcticus*). This fraction resists further alkaline hydrolysis, is precipitable by acetone from petroleum ether, is soluble in dilute aqueous alkali, and is split by an alkaline phosphatase. Ethyl ether then extracts from the very alkaline medium a long-chain α-glycol, which can be demonstrated by the ordinary α-giyceryl ether assay.

Iodine studies in Ascophyllum using radioactive iodine. Sally Kelly and Norman Baily.

On the basis of studies using radioactive iodine, segments of the brown alga, *Ascophyllum nodosum* (Linn.) LeJolis, were found to remove iodine from sea water according to an exchange process. Over a 24-hour period the segments removed iodine at a rapid rate initially, followed by removal at a much slower rate.

The initial rapid removal is interpreted as being due to exchange between iodine in the intercellular spaces and the subsequnt slower removal to exchange between iodine in the cells. That exchange and incorporation into the cells is involved was indicated from experiments using 1) inert solutions, 2) solutions containing various amounts of iodides, 3) hourly replenishment with fresh solutions.

By varying the concentration of iodides in the surrounding medium from 200 to 5000 gamma per liter, it was observed that the concentration of iodine within the segments appeared to be independent of the concentration outside. The concentration of iodine inside the segments could be calculated from the amount of radioactive iodine exchanged, and preliminary calculation indicates an internal concentration of approximately 0.2 mg./g. dry weight. This represents a relative concentration of iodine within the algae about 200 times that of an equal volume of water.

Iodine was found to be taken up in the form of iodides and not as iodates, although the latter is thought to be present in greater concentration in sea water.

That the exchange is characteristic of living protoplasm was demonstrated by the fact that there was no removal of radioactive iodine from solution by dead segments.

The valence of copper in hemocyanin. Martin P. Schulman and George Wald.

It is commonly believed that in hemocyanin, as in hemoglobin, the metal ion remains reduced on oxygenation. Yet, viewed without prejudice, many properties of oxyhemocyanin agree better with the idea that it is a cupric complex. It is blue, a cupric color; and its absorption band at about 575 mμ lies close to those of artificial cupri-protein complexes. The "methemocyanin" supposedly produced by treatment with strong oxidants retains essential properties of hemocyanin itself, including reversible combination with oxygen. The compound allegedly formed by hemocyanin with CO has the spectrum simply of reduced hemocyanin. It might readily be supposed therefore that the combination of hemocyanin with oxygen is a true oxidation, and that in oxyhemocyanin, copper is in the cupric state.

This would make hemocyanin analogous to a copper oxidase. We have inquired into its oxidase activity. Defibrinated Limulus blood oxidizes cysteine, ascorbic acid and catechol with molecular oxygen; tyrosine is not oxidized. This activity, however, resides almost entirely in the serum from which all hemocyanin has been ultracentrifuged. It appears to be due there to catalysis by traces of heavy metal. Hemocyanin itself, purified by ultracentrifugation and washing, has almost negligible oxidase activity with these substrates.

Defibrinated Limulus blood reduces itself, turning colorless on standing. It displays also a measurable oxygen uptake. Purified hemocyanin, however, neither reduces itself nor consumes oxygen. It does both on addition of blood ultracentrifugate or a boiled extract prepared from it.

It is well known that proteins generally yield a violet coloration on treatment with cupric ions and dilute alkali. We have found—and then learned that it has been known since Henze (1901)—that oxyhemocyanin yields this test on simply adding alkali; the hemocyanin itself provides both the protein and the cupric ions. We find, however, that no coloration is obtained on adding alkali to reduced hemocyanin (Limulus). This seems to indicate that copper is cupric in oxyhemocyanin, and cuprous in reduced hemocyanin.

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THE SOURCES AND ACTIVITIES OF TWO CHROMATOPHOROTROPIC HORMONES IN CRABS OF THE GENUS SESARMA. II. HISTOLOGY OF INCRETORY ELEMENTS[1]

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In a previous paper dealing with physiological analyses concerning the activities and possible sources of chromatophorotropic hormones in three Japanese species of *Sesarma* (Enami, 1951), I have shown the sites of origin of two kinds of hormones, namely S and N hormones, on a gross anatomical scale. On the one hand, S hormone was concluded to be produced in both the sinus gland and all the principal ganglia, its occurrence in the latter being independent of the secretory activity of the former. This suggested a strong possibility of the universal existence in ganglionic tissues of a certain incretory mechanism essentially similar to that involved in the sinus gland.

On the other hand, N hormone was demonstrated to be much restricted in its distribution, only a few of the central nervous organs, *i.e.*, the brain and the medulla terminalis in the adults, the brain, the medulla terminalis, the commissural ganglion, and the thoracic ganglion in the juvenile forms, apparently producing the hormone. This suggested the presence in the said ganglia of specific incretory elements not as widely distributed as the presumed sources of the S hormone.

A histological study was carried out in order to determine the ultimate sources of the two hormones as well as the cytological details of their elaboration. The present work also includes a closer investigation of the sinus gland, whose secretory activity has hitherto been studied only superficially, in spite of the generally admitted significance of the gland as the predominant incretory organ in crustaceans.

MATERIALS AND METHODS

Observations were mainly based on histological sections of tissues of the adults of *Sesarma dehaani,* weighing 25–30 g. and showing no sign of ecdysis (*cf.* Enami, 1951). For the purpose of the present study standard sections were prepared from the whole soft eyestalk tissues carefully freed from the exoskeleton, the isolated sinus gland, and the body ganglia such as the brain, the commissural ganglion, and the thoracic ganglion dissected out separately. The tissues were fixed in Heidenhain's Susa for 4 to 8 hours, and paraffin sections 8–10 μ thick were stained with Mallory's triple stain.

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[1] This work was carried out at the Mitsui Institute of Marine Biology, Shimoda, Japan, with the support of a grant-in-aid from the Ministry of Education of Japan in 1948.

For specific purposes, especially for a detailed examination of the sinus gland, various other techniques including both vital and supravital staining were employed, references to which will be made in the following accounts.

Information obtained in the adults of *Sesarma dehaani* was compared with that in the adults of two other species of the same genus, *viz., S. intermedia* and *S. haematocheir,* with corresponding results. Accordingly, the following account applies to all these species.

OBSERVATIONS

Histological signs of an incretory activity in the adults of *Sesarma* were observed in the following tissues and cells:

(1) Sinus gland
(2) Neurilemma of all ganglionic tissues examined
(3) Specific nerve-cells located within certain ganglia. Among these, three types can be distinguished as to their secretory behavior; they are here tentatively designated as α, β, and γ neurosecretory cells, respectively.

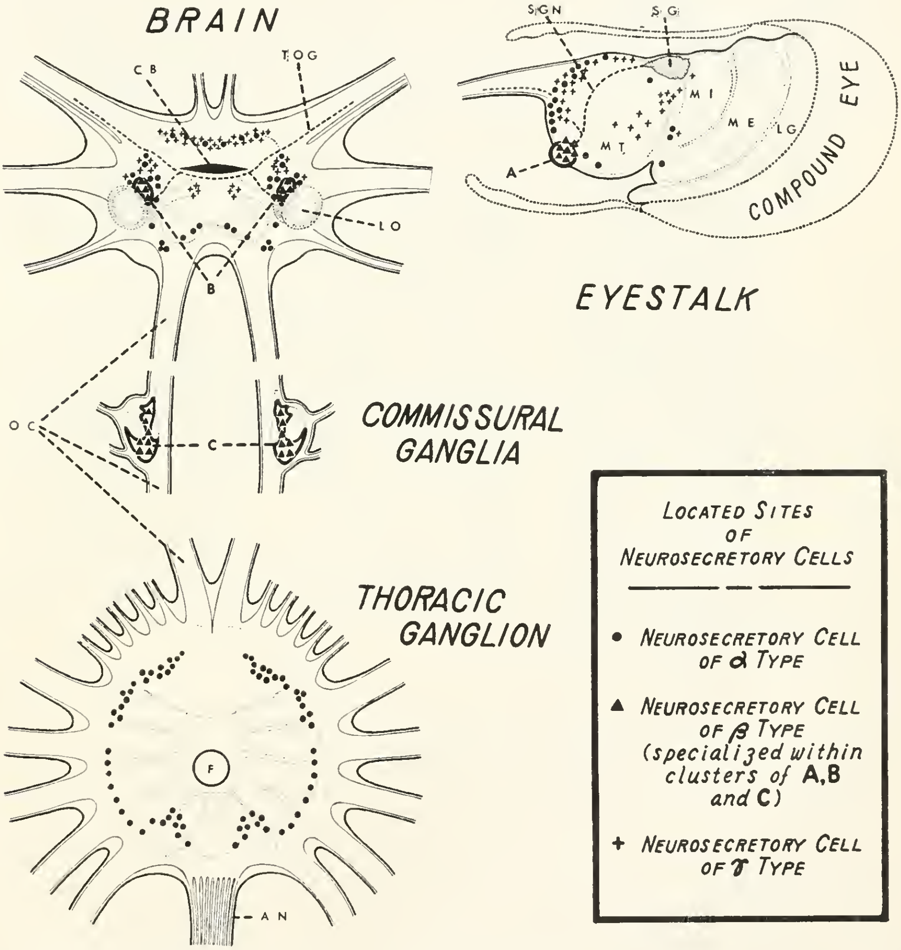
Thus, altogether five kinds of incretory elements are dealt with, their topography being diagrammatically illustrated in Figure 1.

1. The sinus gland and its secretory behavior

The sinus gland of *Sesarma* is well developed and, in fresh or even in formalin-fixed eyestalks, is macroscopically visible owing to its well-known characteristic opacity and slightly bluish hue (Brown and Cunningham, 1939, etc.). It is located at the dorsal aspect of the junction between the medulla interna and the medulla terminalis (Fig. 1, SG). Its outline roughly conforms to a triangle, one of whose apices is tapering off in a proximal direction. When viewed through transmitted light, the gland appears brownish yellow in marked contrast to the almost colorless nervous tissues nearby. The approximate volume of the gland was measured in animals of different sizes by means of a simple calculation from the area of optical section and the thickness of the isolated gland sandwiched between the slide and cover glass of a Thoma-Zeiss hemacytometer (Fig. 2).

The proximal tapering indicates the innervation by the sinus-gland nerve which is traceable along the frontal surface of the neuropile of the medulla terminalis up to a disc-like cluster of giant nerve-cells located at the proximal ventral-lateral corner of the medulla (Fig. 1, A). The nerve leaves the nerve-cell group at the center of the latter's internal concavity, and within a short distance from the point of its departure sends off a slender branch proximally, which enters into the pedunculus lobi optici (Fig. 1). Concerning the innervation of the gland in *Cambarus,* Welsh (1941) has described, besides the unquestionable innervation by the sinus-gland nerve originating in the medulla terminalis, a distribution to the surface of the gland of a branch of the oculomotor nerve II, a fact which demonstrates a dual innervation of the gland. However, as far as the present study is concerned, the innervation of the sinus gland of *Sesarma* takes place by the sinus-gland nerve exclusively, no other routes having been found, although some of the finer branches of the oculomotor nerve II, in supravital staining with methylene blue, appear to be in superficial contact with the glandular tissue.

The glandular tissue is, just as described in the sinus glands of various other crustaceans (Hanström, 1933, 1937; Sjögren, 1934; Ståhl, 1938; Carstam, 1941; Pyle, 1943; etc.), composed of a folded continuous lamella of syncytium, including a variable amount of large and small acidophile droplets. Several branches of the dorsal radial blood-sinus are enclosed, a continuous structureless membrane of about 0.3 μ thickness separating the glandular lamella from the blood-sinus (Fig. 3, L.M.). This limiting membrane was made visible in deep blue color by means of Mallory's triple staining after fixation with 5% sulfosalicylic acid. It is noteworthy that,


BRAIN
CB
TOG
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B
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MT
A
COMPOUND EYE
EYESTALK
OC
C
COMMISSURAL GANGLIA
THORACIC GANGLION
F
AN
LOCATED SITES OF NEUROSECRETORY CELLS
● NEUROSECRETORY CELL OF α TYPE
▲ NEUROSECRETORY CELL OF β TYPE (specialized within clusters of A, B and C)
+ NEUROSECRETORY CELL OF γ TYPE

FIGURE 1. Diagram of the central nervous organs and the eyestalk tissues of *Sesarma* showing distribution of incretory elements. AN, abdominal nerve; CB, central body of the brain; F, foramen; LG, lamina ganglionaris; LO, lobus olfactorius; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OC, oesophageal commissure; SG, sinus gland; SGN, sinus-gland nerve; TOG, tractus olfactorio-globularis.

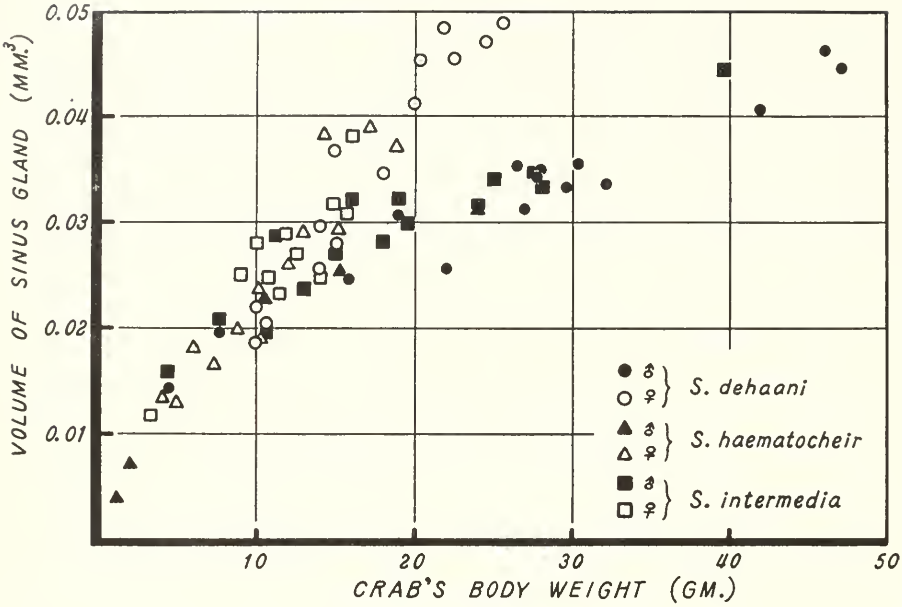

FIGURE 2. Volume of the sinus gland plotted against the body weight in three species of *Sesarma*.

contrary to Hanström's statement pointing out the perforated nature of the membrane (1937), I observed the membrane as being complete everywhere along its course in good sections. I repeatedly examined the membrane in a number of preparations with the result that what had been dealt with as perforations might have been one of the artefacts frequently encountered in sections as those prepared from the whole eyestalk enclosed by hard exoskeleton, to which Hanström had resorted.

At the opposite side of the limiting membrane, the tissue of the sinus gland is contiguous with surrounding spongy connective tissue (Fig. 3, CT), a landmark between the tissues being represented by sets of coarse networks of fibers from the sinus-gland nerve (Fig. 3, E.B.).

Thus, the sinus gland of *Sesarma* is supplied with innervation from the outside and encloses the blood-sinus in the interior, which indicates that it belongs to the so-called inverse type of Hanström's classification (1937, 1947a, etc.).

The tissue of the sinus gland is remarkably similar in its fundamental structure to the neurilemma of nervous tissues, a fact long known by workers of Hanström's school and now substantiated by observations with preparations in which, by treatment with 5% hydrochloric acid prior to fixation, the sinus gland was freed from acidophile inclusions that mask the details of basic structure. The lamella of the gland is, as stated above, essentially of syncytial nature, being composed of continuous chromophobe hyaloplasm divided into irregular units by incomplete septa consisting of coalesced and half-coalesced granules (Fig. 3, S). Within each of the units are a variable number of nuclei of different appearance. Such characteristics are in complete accord with those observed in the neurilemma tissue (Fig. 4). Surrounding the blood-sinus, units of the sinus-gland syncytium are cylindrical, standing vertically against the limiting membrane (Fig. 3).

A remarkable difference between the sinus gland and the neurilemma exists in regard to the amount and morphological variability of the acidophile inclusions, the sinus gland predominating in these respects. As to configuration, stainability, plasticity, microscopic composition, etc., three types of inclusions within the sinus gland are distinguishable which are here tentatively dealt with as colloids A, B, and C, respectively.

Colloid A (Fig. 3, middle figure, CA). Ovoid or ellipsoid masses ranging from 4 μ to 8 μ in length, stained most intensely with the acid fuchsin component of Mallory's triple stain. It is found either in isolation or in small aggregations, its

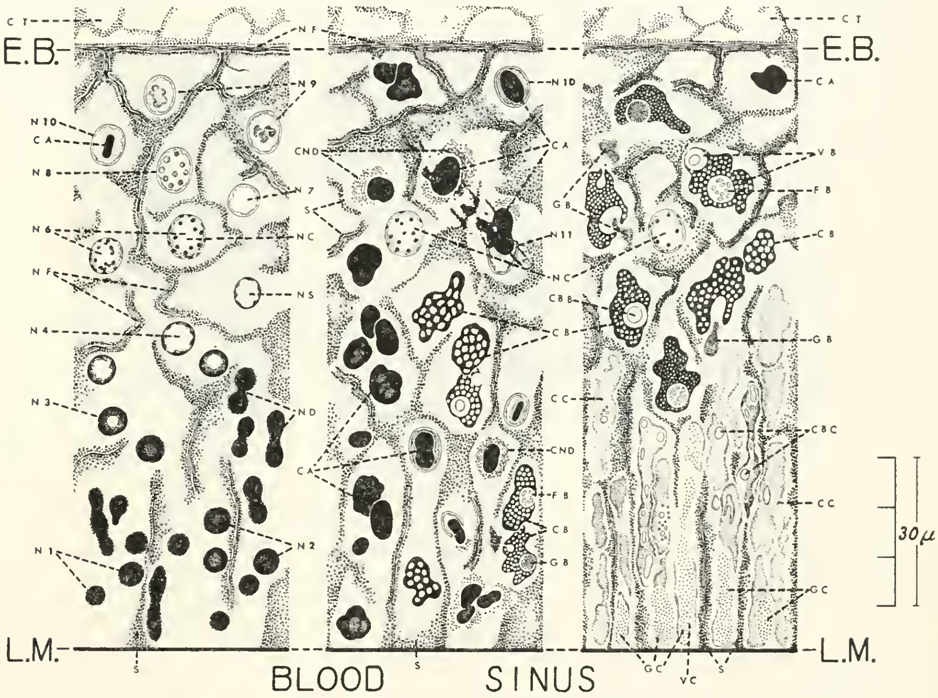

FIGURE 3. Histology of the sinus gland illustrated semi-diagrammatically on the basis of studies with sections and supravital mounts. The whole process of secretory behavior is, for the sake of convenience, arranged in three separate views, of which the left one represents the process of transformation of the nucleus, and both the middle and the right ones are indicating the process of transformation of secretory precursor derived from the nuclear change. E.B., external boundary of the glandular tissue; L.M., limiting membrane.

Left figure: N 1–N 10, successive states of nuclear change; NC, nucleus most commonly seen in the gland; ND, nucleus showing direct division; CA, colloid A formed within the nucleus; NF, nerve-fibers; CT, connective tissue; S, incomplete septum of syncytium.

Middle figure: N 11, liberation of colloid A from nuclear capsule; CND, nuclear capsule in the process of disintegration; CB, colloid B; FB, fragmentary inclusion in the vacuole of colloid B; GB, intra-vacuolar granules of colloid B; other notations are the same as those in the left figure.

Right figure: VB, vacuole in colloid B; CBB, central body in the vacuole of colloid B; CC, colloid C; CBC, central body in the vacuole of colloid C; GC, granules in the vacuole of colloid C; VC, vacuole in colloid C; other notations are the same as those in the left figure.

occurrence being inconsistent at different spots of the same glandular lamella. In the supravital state the colloid shows a marked affinity to alizarine S, the resulting coloration being associated with pronounced refractility, and it displays considerable plasticity, which suggests a high viscosity of its composite material. In both fixed and *in vitro* preparations, its staining is homogeneous, no structural differentiation being present. Characteristic staining reactions of the inclusions are further observed in sections impregnated with silver according to the techniques of Bielschowsky and of Hortega, in which the colloid reduces silver almost selectively. In sections treated with $N/4$ sodium hydroxide and stained with Janus green B, the colloid appears deep greenish blue in contrast to the unstained surrounding tissue elements.

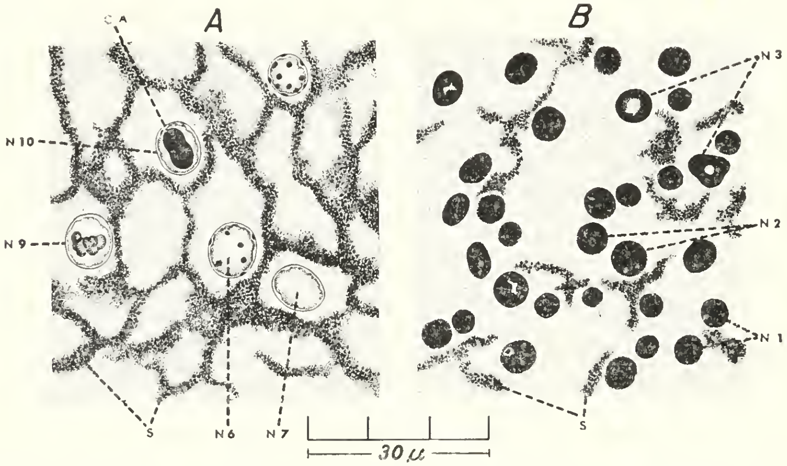

FIGURE 4. Histology of the neurilemma semi-diagrammatically illustrated. A—Portion of the neurilemma showing formation of colloid A within the nucleus. B—Portion of the neurilemma including a crowd of small basophile nuclei. Notations are the same as those in Figure 3.

Colloid B (Fig. 3, middle and right figures, CB). Irregular-shaped masses seemingly formed by coalescence of masses of colloid A. In comparison with the latter, it shows weak stainability with both acid fuchsin in Susa-Mallory preparations and alizarine S applied *in vitro*. Together with the lowering of the affinity to these dyes, a marked decrease in the power of silver reduction together with the stainability with Janus green B is characteristically observed with this kind of glandular inclusion. In contrast to the apparent homogeneity of colloid A, colloid B is differentiated into a feebly acidophile matrix substance which appears less viscous than colloid A, and almost chromophobe contents of various sizes and amounts. Vacuoles occur in the colloid (Fig. 3, right figure, VB), which remain achromatic in Susa-Mallory preparations but are selectively stained with trypan blue, anilin blue, Congo red, eosin and tropeolin *in vitro*. They may or may not include formed elements, whose appearance ranges from single large chromophobe bodies (Fig. 3, right figure, CBB) to several irregular achromatic fragments (Fig. 3, middle and right figures, FB) and a number of granules deeply stained with anilin blue in Susa-Mallory sections (Fig. 3, middle and right figures, GB). Such vacuolar inclusions are unstainable *in vitro,* but are clearly demonstrated when their surroundings are stained with the dyes mentioned above. Irrespective of their size, no sign of Brownian movement is observed *in vitro,* which is suggestive of considerable viscosity of their surroundings within the vacuoles. It was not infrequent to observe in both sections and supravital mounts of the sinus gland that the said anilin-blue granules together with their surrounding viscous substance were on the way of freeing themselves from colloid B into the hyaloplasm of the glandular tissue (Fig. 3, right figure).

Colloid C (Fig. 3, right figure, CC). Almost formless masses which, by coalescence, grow to larger forms conforming to the contours of the divisions of the glandular tissue. In Susa-Mallory preparations, this kind of inclusion is stained fairly well and homogeneously with Orange G, no morphological differentiation of composition being obvious. In whole mounts of the sinus gland *in vitro,* a wide affinity of the colloid to basic dyes such as Dahlia, Cresylecht violet, Gentian violet, methylene blue, and neutral red was disclosed, and an evident sign of its low viscosity was its tendency to assume a rounded appearance. In contrast to both colloid A and colloid B, this colloid rejects alizarine S *in vitro,* and in sections it reacts negatively to silver impregnation and also to staining with Janus green B following treatment with NaOH. Moreover, it is characterized by active vacuole formation (Fig. 3, right figure, VC). In vacuoles of various sizes one finds a number of granules of uniform size (Fig. 3, right figure, GC), several irregular fragments, or one or several central bodies (Fig. 3, right figure, CBC), all of which remain unstainable in both fixed and supravital conditions. *In vitro,* such vacuolar inclusions exhibit more or less pronounced Brownian movement, the smaller their size, the more active the visible motion. This fact indicates a certain fluid nature of the vacuolar content surrounding the inclusions. The development of such vacuoles in this colloid is very pronounced, and there are figures indicating that a given mass of the colloid may be almost wholly occupied by an enlarged vacuole.

Possible transformation of secretory materials within the sinus gland. The features described above are characteristic of the three representative forms of colloid inclusions of the sinus gland, but there are other forms derived from the respective colloids, which lie either between colloids A and B or between colloids B and C. This fact indicates a possible continuity of all the colloidal inclusions, *i.e.,* the possibility of a transformation of colloid A to colloid C by way of colloid B. In relation to such a possibility, the following observation appears to be of interest.

Upon treatment with 10% pyridine, a freshly isolated sinus gland loses most of its characteristic opaque whiteness in less than 30 minutes. Formalin-fixed and Mallory-stained sections of such material reveal that the vanishing of whiteness is due to the almost complete disappearance of colloid C. Nevertheless, both colloid A and colloid B are known to be resistant to the treatment. Of these remaining inclusions, colloid A as a whole retains a remarkable affinity to acid fuchsin, while colloid B reacts to the dye in a manner different from that shown by the one not treated with pyridine. The fragmentary inclusions within colloid B take the dye almost selectively, whereas the homogeneous ground substance around them is but feebly stained. That is, the affinity to acid fuchsin is reversed in the two kinds of components of colloid B as a result of the treatment with dilute pyridine.

After all, there are indications that the initially highly viscous and homogeneous mass (colloid A) differentiates into a less viscous substance as the result of progressive disintegration into finer particles, which further changes into a more fluid substance as the result of a separation of viscous substance by means of vacuole formation. The fluid substance thus produced (colloid C) may then change into intravacuolar fluid and granules, both of which appear to be the ultimate products of transformation of colloid A, taking place within the tissue of the sinus gland.

Derivation of secretory material from nucleus. Inasmuch as the acidophile inclusions of the sinus gland are considered as secretory material (*cf.* Hanström, 1937, etc.), the preceding account of the possible fate of colloid A throws some light on the process of formation of the active material. Another problem, so far left untouched, concerns the origin of the glandular inclusions.

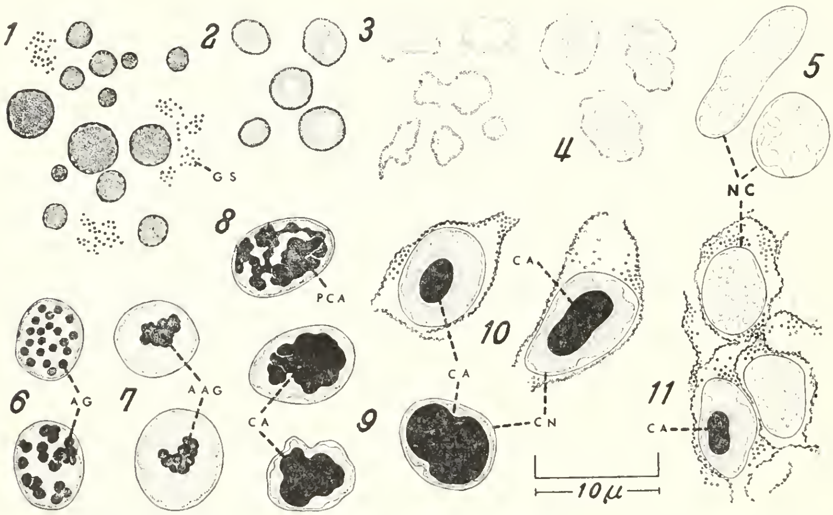

FIGURE 5. Variety of nuclear figures in both the sinus gland and the neurilemma. Susa, Mallory. NC, common appearance of nucleus; GS, granules of septum of syncytium; AG, globules stained with Orange G; AAG, aggregated Orange G-globules; PCA, precursor of colloid A; CA, colloid A; CN, capsule of nucleus surrounding colloid A.

It was not infrequent to observe in both sections and supravital mounts of the sinus gland that colloid A in its isolated form was enclosed by an ovoid or ellipsoid capsule of considerable thickness which, although being refractory to any dyes, is visible on account of its high refractility (Fig. 3, left and middle figures, N 10; Fig. 5, *10*). Some figures showed the release of the colloid masses from their respective capsules through rupture of the latter (Fig. 3, middle figure, N 11; Fig. 7, *2–5*). In areas of the glandular lamella where such figures were found in close aggregation, freed masses of the colloid were seen in coalescence with each other to form larger masses which were associated with empty capsules (Fig. 7, *1*). The capsule, after releasing the colloid mass, appeared to disintegrate, being at first decomposed into irregular pieces and eventually reduced to finer granules (Fig. 3, middle figure, CND; Fig. 7, *6–9*).

In the state of full integrity, the encapsulated body conforms in size and form to the nucleus, a fact which led me to an inquiry regarding the role of the nucleus of the sinus gland. In the routine hematoxylin-eosin preparations, the nuclei of the gland most commonly met with are of vesicular appearance, containing varying amounts of basophile granules which may be taken for granular chromatin, but no acidophile elements corresponding to nucleoli. In sections treated after Feulgen's method modified by Rafalko (1946),[2] it was found that the chromatin or, more precisely, the basophile elements are arranged at the periphery of the nucleus, the latter's interior being occupied by chromophobe caryoplasm (Fig. 3, NC or N 6; Fig. 6, *7*). Besides this appearance there were other features of the nucleus, indicating its derivation from a smaller structureless basophile body.

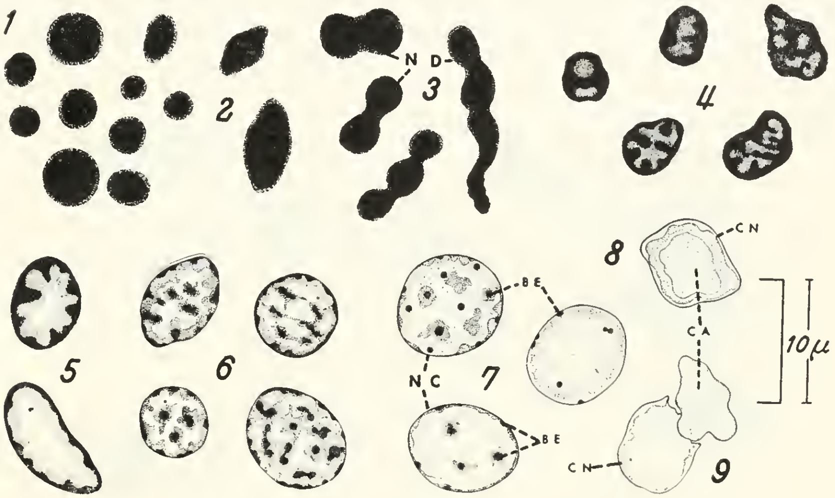

FIGURE 6. Figures of nuclei in both the sinus gland and the neurilemma. Helly, Feulgen (Rafalko's modification). ND, amitotic figures of nuclear division; NC, common appearances of nuclei. BE, basophile elements at the periphery of nucleus; CA, colloid A; CN, capsule of nucleus.

What is designated to be the original form of the nucleus is a minute sphere, ovoid or ellipsoid, of dimensions ranging from $3\,\mu$ to $6\,\mu$, which stains with hematoxylin, safranin and muci-carmine and also exhibits a marked uniform reaction with Feulgen's stain except for a thin peripheral area (Fig. 3, left figure, N 1; Fig. 6, *1* and *2*). In Susa-Mallory preparations, such a body takes anilin blue uniformly, but the peripheral area predominates in this stainability (Fig. 5, *1*). Nuclei of such structureless appearance were at times found in dense aggregation in certain restricted areas of the sinus gland, where they were observed in association with groups of melanin granules, and occasionally in the process of direct division (Fig. 3, left figure, ND; Fig. 6, *3*).

Such a state of the nucleus appears to change to a state in which the chromophobe portion is differentiated in the center of the basophile mass (Fig. 3, left figure, N 2 and N 3; Fig. 5, *2*; Fig. 6, *4*), and there were figures showing further successive changes, in which the nucleus becomes enlarged by the expansion of the central chromophobe mass which is likely to take place at the expense of the basophile portion restricted to the periphery (Fig. 3, left figure, N 4 and N 5; Fig. 5, *3* and *4*; Fig. 6, *5* and *6*). These changes seem to result in the common vesicular state of the nucleus already referred to.

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[2] Use of this technique was aided by the kind information received from Dr. K. W. Cleland, Sydney University, Australia.

Figures were observed indicating the formation of strongly acidophile droplets within the chromophobe substance of the nucleus, which stained with the Orange G component of Mallory's stain and in this respect resembled the ordinary nucleoli (Fig. 3, left figure, N 8; Fig. 5, *6*). In some of the nuclei, such droplets showed a marked tendency to coalesce (Fig. 3, left figure, N 9; Fig. 5, *7*), and along with this phenomenon the refractile achromatic capsule became evident. It appears probable that colloid A is formed as the result of such changes (Fig. 5, *8*), accompanying the formation of its capsule, but a decision concerning this point cannot be reached at present because of the lack of definitive observations.

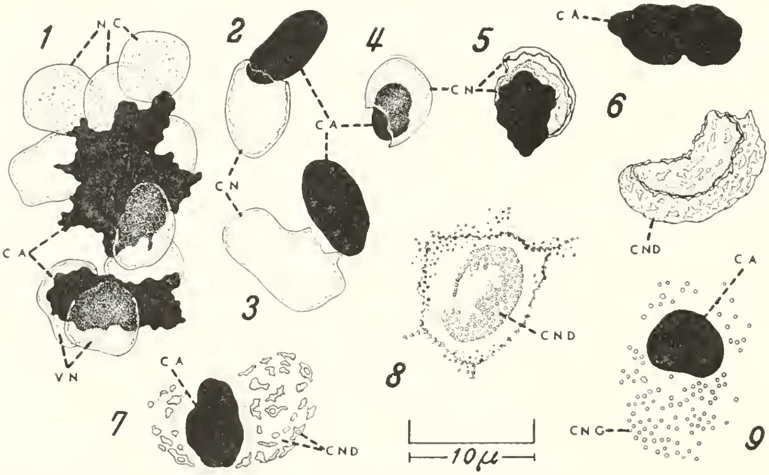

FIGURE 7. Figures showing liberation of colloid A from the nucleus and the later fate of nuclear capsule in the sinus gland and the neurilemma. Formalin, *N*/4 NaOH, Janus green B. NC, common forms of nuclei; CA, colloid A; VN, vacant nuclei; CN, capsule of nucleus; CND, disintegrating capsule of nucleus; CNG, granules resulted from disintegration of nuclear capsule.

2. *Secretory activity of the neurilemma*

Just as in the sinus gland, the nucleus plays a significant role in the production of acidophile inclusions in the neurilemma. The neurilemma of all ganglionic tissues was observed to produce a kind of colloid which was morphologically quite similar to colloid A of the sinus gland (Fig. 4, *A*, CA). In Susa-Mallory preparations or more clearly in sulfosalicylic acid-Mallory preparations of each of the principal ganglia, the colloid in question was found in varying amounts in different portions of the neurilemma, whose brilliant red coloration stood out impressively from the light blue tinge of the background. It was further observed that what was known about the morphological change of the nucleus within the sinus gland in relation to the process of the formation of colloid A was also the case regarding the nucleus of the neurilemma (Fig. 4). Such facts indicate that the same secretory mechanism is operating in the two kinds of tissue in question, which, as stated above, are remarkably similar in their fundamental structure.

However, the neurilemma was observed to be inferior to the sinus gland with respect to the nuclear activity, and, moreover, present investigation failed to ascertain the transformation of colloid A into other forms, because of the apparent absence of both colloid B and colloid C or their homologues within the neurilemma tissue.

3. The neurosecretory cells

The present study includes the search for possible incretory elements in the nervous tissues outside the neurilemma, which, as already stated, resulted in the finding of three kinds of neurosecretory cells.

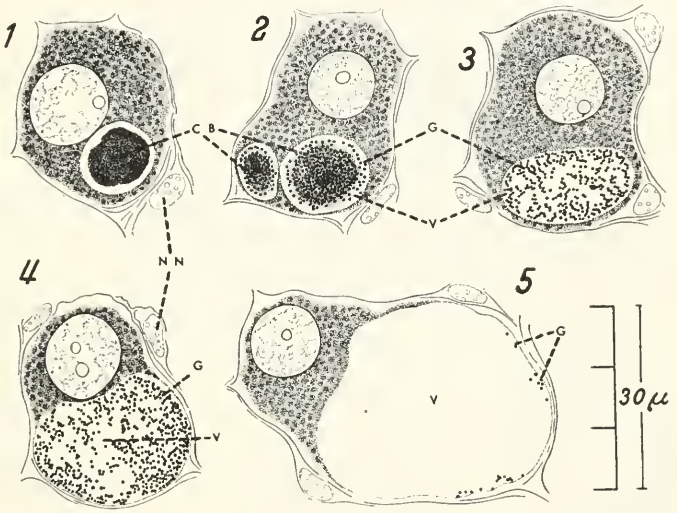

FIGURE 8. α neurosecretory cells from the brain and the medulla terminalis. Susa, Mallory. CB, central body formed in vacuole; G, granules derived from the central body; V, vacuole; NN, nucleus of the neurilemma.

α *neurosecretory cell* (Fig. 8). Secretory behavior was observed in some unipolar nerve-cells of the common type widely distributed in the brain, the medulla terminalis, the medulla interna and the medulla externa as well as the thoracic ganglion (Fig. 1). Excepting those found in the thoracic ganglion which were represented by giant cells attaining about 60 μ in length and including a large vesicular nucleus of about 20 μ diameter with one or two nucleoli of about 4 μ, the cells in other ganglionic tissues are generally of moderate size, measuring about 30 μ in length and including a nucleus of about 12 μ in diameter in which one or two nucleoli of about 2 μ are present. Irrespective of the difference in cellular dimensions, all of the cells are rich in cytoplasm, in which significant vacuole formation takes place. In Susa-Mallory preparations of the said ganglia, formation of a large fuchsinophile body within each vacuole (Fig. 8, *1*), splitting of the body into a number of fuchsinophile granules (Fig. 8, *2*), and dispersion of isolated granules within the largely expanded vacuole (Fig. 8, 3 and 4) were seen. Sometimes, expanded vacuoles were observed to be almost free of formed inclusions except for a few fuchsinophile granules (Fig. 8, 5). With the formation of such vacuoles, the cytoplasm of the cells exhibited a variable affinity to acid fuchsin; the cytoplasm rejected the dye in the presence of many fuchsinophile granules within the vacuole, but was

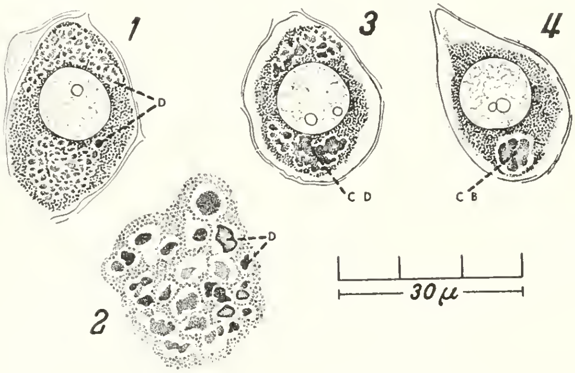

FIGURE 9. β neurosecretory cells from the brain and the commissural ganglion (*cf.* Fig. 1, B and C). Susa, Mallory. D, droplets appearing within cytoplasm; CD, droplets coalesced to form homogeneous mass; CB, central body formed. Scale is applicable to 1, 3, and 4. 2 is a further magnification of portion of cytoplasm in 1.

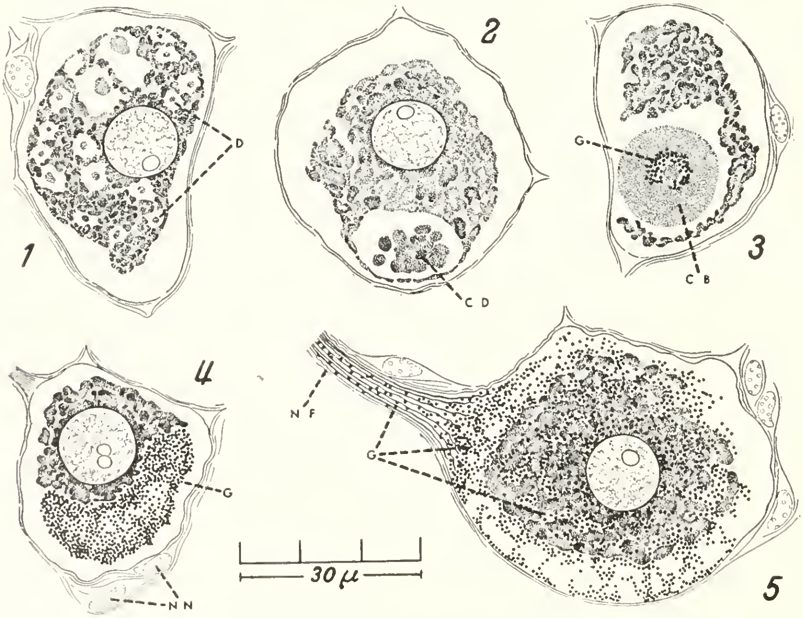

FIGURE 10. Giant β neurosecretory cells from the medulla terminalis (*cf.* Fig. 1, A). Susa, Mallory. NF, nerve-fiber innervating the sinus gland; NN, nuclei of the neurilemma; G, secretory granules; other notations are the same as those in Figure 9.

stainable in higher or lower degrees when the vacuole was deprived of most of its granular inclusions. Pictures were observed indicating a possible absorption of the intra-vacuolar granules by the cytoplasm, a fact which might explain the fuchsinophilic character of the cytoplasm during the disappearance of the granules.

β neurosecretory cell (Figs. 9, 10, and 11). The second type of neurosecretory cells differs from the preceding one in its mode of secretory activity and in the predominant affinity of the secretory material to the anilin blue component of Mallory's stain and also in the mode of secretory behavior. Cells showing this type of secretion were detected in the brain, the medulla terminalis and the commissural ganglion, but not in other ganglia. In the brain, such cells were located exclusively in a group of nerve-cells situated just anterior to the lobus olfactorius (Fig. 1, B). They, together with those found in the commissural ganglion (Fig. 1, C), are of moderate size, measuring ca. 30 μ in length and including a vesicular nucleus of ca. 10 μ in diameter (Fig. 9). Their cytoplasm is fairly homogeneous and of compact appearance, showing but slight contraction upon fixation. The cells belonging to the medulla terminalis are represented by a number of giant unipolar cells which constitute the conspicuous cluster at the ventral surface of the proximal portion of the neuropile of the medulla, dispatching nerve-fibers innervating the sinus gland

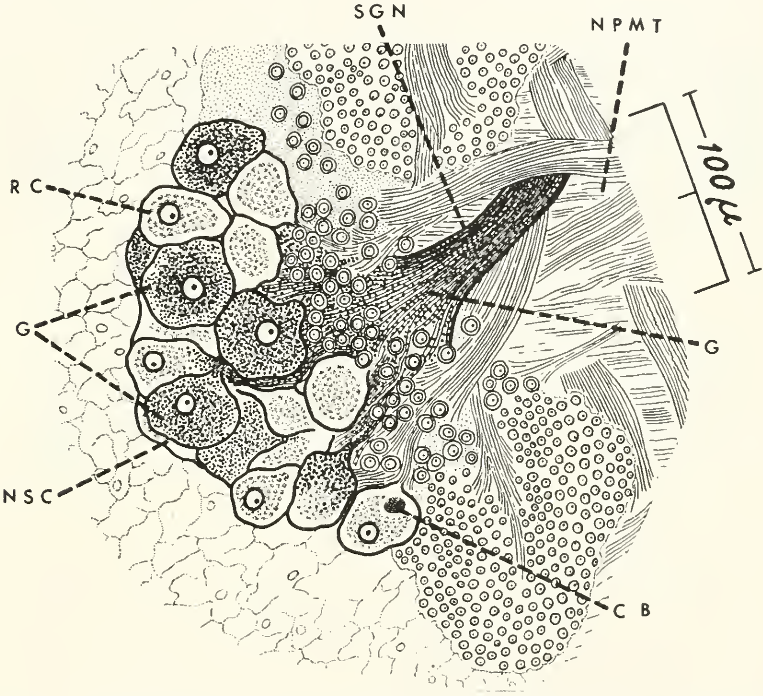

FIGURE 11. Section of the cluster of giant β neurosecretory cells of the medulla terminalis (*cf.* Fig. 1, A). Susa, Mallory, sagittal to axis of the eyestalk. SGN, sinus-gland nerve; NPMT, neuropile of the medulla terminalis; G, secretory granules; RC, resting cell; NSC, cell in secretory activity; CB, central body formed in the cell.

(Fig. 1, A; Fig. 11). These cells are composed of loose cytoplasm which, upon fixation with Bouin, Allen-Bouin, formalin, Helly, sulfosalicylic acid, Susa, and Zenker, contracts more or less conspicuously. The average dimension of the cells, measured by the contour of the surrounding neurilemma capsule, was about 50 μ, and the nucleus attained about 15 μ in diameter (Fig. 10).

In the cytoplasm of the β-type neurosecretory cells occurred many droplets feebly stained with anilin blue (Fig. 9, *1* and *2*; Fig. 10, *1*). Also observed in the cytoplasm of some cells were one or two spherical bodies taking up anilin blue considerably, which seemed to result from coalescence of the said droplets (Fig. 9, *4*; Fig. 10, *3*; Fig. 11, CB). Each of these spherules was observed to split into fine granules stained deeply with the dye (Fig. 10, *3* and *4*). The resulting granules were dispersed over the whole area of the cytoplasm, and it was of much interest to find in a few instances that they were present also along the path of axons arising from the cells with secretory activity (Fig. 10, *5*; Fig. 11, G). In one case, the granules were detected along the course of the sinus-gland nerve for approximately two-thirds of its whole length.

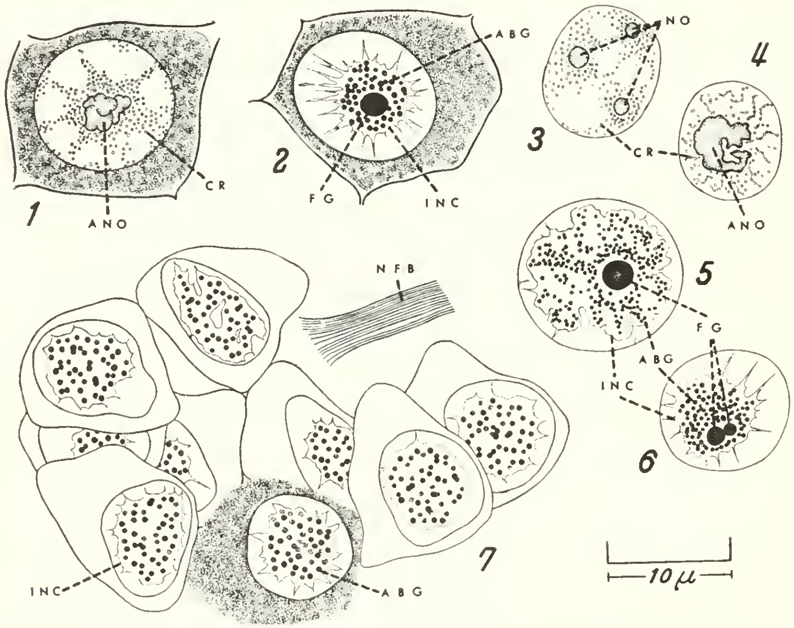

FIGURE 12. γ neurosecretory cells from the brain and the medulla terminalis. Susa, Mallory. 3–6 show nuclei only; 7 shows a cluster of the cells indicating synchronism of nuclear activity. ANO, nucleoli aggregated at the center of nucleus; CR, chromatin granules; FG, fuchsinophile globule; ABG, anilin-blue granules; INC, intra-nuclear colloid; NO, nucleoli; NFB, nerve-fiber bundle.

γ neurosecretory cell (Fig. 12). Unlike the preceding two types of neurosecretory cells, the third type is characterized by a kind of nuclear secretion. In some small unipolar nerve-cells measuring about 20 μ in length, as well as in some of the cells belonging to the so-called globulus type, measuring about 10 μ in length, the nucleus underwent a series of changes unassociated with any visible change in the cytoplasm. There were pictures showing formation of a large intra-nuclear droplet uniformly stained with the Orange G component of Mallory's stain, within some of which were differentiated a fuchsinophile spherule and many anilin blue granules (Fig. 12, *2* and *5*). The fuchsinophile spherule was different from the nucleolus in the ordinary state of the cells in question in that it was far larger than the latter and was stained conspicuously with acid fuchsin in contrast to the latter's affinity to Orange G. Similarly the anilin blue granules were different from the granular chromatin in size and in stainability, the former far exceeding the latter in this respect. Other figures showed that both the fuchsinophile spherule and the anilin blue granules were reduced to smaller forms. That is, two or more globules developed from a single spherule, which, unlike their preceding form, took up Orange G considerably and resembled nucleoli, and a number of fine granules were derived from the anilin blue granules, which resembled the chromatin (Fig. 12, *6*). These figures are suggestive of a reduction of intra-nuclear inclusions to nucleoli and chromatin granules. As regards the process of formation of the intra-nuclear colloid, there were indications suggesting its origin from a central aggregation of both the nucleoli and the chromatin (Fig. 12, *1, 3,* and *4*).

Such being the case, it would appear plausible that the nucleus of this type of nerve-cell shows a kind of reversible change, and that the secretion of a diffusible substance takes place in the course of the change. The present study failed to obtain any sign of discharge of formed elements from the nucleus to the surrounding cytoplasm of the cell.

Nerve-cells showing such nuclear change were located in abundance in the brain as well as in the medulla terminalis, but were scarcely detected in other ganglia (Fig. 1).

CONSIDERATIONS ON POSSIBLE SOURCES OF CHROMATOPHOROTROPIC HORMONES

On the basis of the histological findings so far described, possible sources of the two chromatophorotropic hormones of *Sesarma* may be analyzed.

Taking into account the results of the experimental study (Enami, 1951) which have demonstrated that one of the hormones, S hormone, is originating not only in the sinus gland but in all the principal ganglia in the adults, one may attach some significance to the remarkable identity of the fundamental structure as well as the mode of secretion of the sinus gland and the neurilemma universally associating with the nervous tissues. These structures are equivalent in that their secretions appear to originate as the result of a transformation of the nucleus, although the fate of the secretory material within the respective tissues appears to be different. The secretory precursor, or colloid A, in the sinus gland undergoes a sequence of complicated changes to be transformed into a semi-fluid form (colloid C) which is further transformed through vacuole formation into liquid and granules. Now, in connection with the problem of what form the final secretion may assume, the observed completeness of the limiting membrane is to be taken into consideration. It appears possible that the final secretion containing S hormone is of diffusible nature, to which the limiting membrane is permeable, and that no kind of formed element can take its way out through the membrane, in which no sign of microscopic perforation was observable. In this respect, the vacuolar fluid of colloid C rather than the co-existing granules appears to be of significance.

Concerning the role of the vacuolar contents of colloid B, which are liberated as a whole from the latter, no concrete information could be obtained. They may somehow be responsible for the formation of certain secretory materials, or they may be nothing but by-products of the transformation of the colloid into less viscous colloid C, having no direct relation to the formation of secretory products.

At any rate, such complicated secretory behavior was not found in the neurilemma, which fact may be correlated with a presumably simpler mode of hormone production in this tissue.

Recent investigations by several authors have demonstrated that the crustacean sinus gland produces various kinds of hormones in addition to the generally admitted chromatophorotropic one, such as that acting on the motility of the heart, the hormone inhibiting molting, the diabetogenic principle, the one inhibiting ovarian growth, the hormone controlling retinal pigment migration and that controlling general motor activity. With the exception of the chromatophorotropic hormone, all of these principles seem to originate in the sinus gland exclusively (*cf.* Brown, 1944; Hanström, 1947a). While it remains to be seen whether such hormones are different substances or represent only one or a few substances, it should be taken into account that there is no concrete information concerning the capacity of the neurilemma for such manifold production of hormones. It may tentatively be proposed that the observed simplicity of the secretory behavior in this tissue appears to be correlated with a restricted productivity of hormonal substances.

With respect to the histological elements responsible for the production of another kind of chromatophorotropic hormone, N hormone, experimental results have indicated that such might be located in the adult crabs in the brain and the medulla terminalis. Such a restriction in the distribution of this hormone points to incretory elements specifically present within the said two nervous organs as the possible sources. Therefore, the neurilemma universally associating with the nervous tissues is to be excluded. A marked parallelism exists between the distribution of the neurosecretory cells of the γ type and the experimentally ascertained sites of origin of N hormone.

As for the other types of neurosecretory cells, there is little possibility of their participation in the production of N hormone. This might be deduced from the experimental results demonstrating the absence of the hormone in concentrated extracts of such ganglia as the thoracic ganglion, in which the cells of the α type are present in greatest abundance, and the commissural ganglion containing the β type cells. Absence of the hormone was also demonstrated in the extract of the isolated cluster of β neurosecretory cells of the medulla terminalis.

It is of much interest that, as demonstrated above, neurosecretion of the γ type is characterized by a kind of nuclear secretory activity, which has also been reported in certain neurosecretory cells as well as in certain hypophyseal cells in some vertebrates (Scharrer, 1934; Scharrer and Scharrer, 1945; Hanström, 1947b). Thus, the production of N hormone can be regarded as being in principle identical with that of S hormone, inasmuch as both involve nuclear activity, even though the mode of nuclear behavior differs. S hormone appears to take its origin at the expense of the nucleus, whereas N hormone results from a reversible change of intra-nuclear elements, during which the nuclear integrity remains intact.

Thus, the chromatophorotropic hormones in *Sesarma* are elaborated by two kinds of nuclear secretion, an observation which contributes further to the recently increasing knowledge regarding the participation of the nucleus in secretory activities in both the vertebrates and the invertebrates. As Palay (1943) has supported the concept of nuclear secretion against the former negative view of Bowen (1929), present work also demonstrates it in certain secretory activities.

Concerning the neurosecretion of the α and β types, no information about their physiological significance is available at present. Of interest is the role played by the secretory material of the β neurosecretory cells, especially those supplying nerve-fibers to the sinus gland. As reported above, the anilin blue granules interpreted as the final product of secretory activity of the cells were observed to take their way along the path of the axon toward the sinus gland, a fact which is reminiscent of similar figures demonstrated by Scharrer and Scharrer (1944) and Palay (1945) in the preoptico-hypophyseal tract in some vertebrates and also in the intercerebralis-cardiacum-allatum pathway in some insects. It appears plausible that the neurosecretion in question may play a significant role in the control of the secretory activity of the sinus gland. However, the present study is short of data for such considerations, except for the following. In an experiment, the nerve-cell cluster in question was carefully isolated from the medulla terminalis and extracted to be injected into eyestalk-less specimens of juvenile *Sesarma haematocheir* and eyestalk-less *Paratya compressa,* with the result that a considerable effect of S hormone (pigment concentration in reddish chromatophores in both recipients; *cf.* Enami, 1951), but not of N hormone was produced. However, whether this result is attributable to a temporary promotion of secretory activity in the recipients' sinus gland brought about by the secretory product contained in the extract, or whether it is due to the direct effect of the donor's S hormone derived from the neurilemma around the nerve-cell cluster in question, is left undetermined.

SUMMARY

1. In this part of my contemplated series of papers are reported some results concerning the histological investigation of the incretory elements responsible for the production of the two chromatophorotropic hormones in *Sesarma,* whose activities and distribution were experimentally dealt with in the preceding study.

2. On the basis of the present histological findings, and their comparison with the experimental data, it has been concluded that the sinus gland and the neurilemma are responsible for the secretion of S hormone, whereas N hormone has its origin in a type of neurosecretory cell (γ neurosecretory cell) occurring in abundance in both the brain and the medulla terminalis.

3. The secretory activity in all of these hormone sources is alike in that it shows nuclear secretion, but there are differences in the secretory behavior.

4. The close relationship between the sinus gland and the neurilemma was substantiated on the basis of their fundamentally similar histological structure and their secretory behavior. However, certain differences were observed as to the fate of the secretory material derived from the nucleus, a more complicated sequence of transformations taking place in the sinus gland than in the neurilemma. The sig-

nificance of these differences and the possible nature of the final secretory material of the sinus gland were discussed.

5. Besides the cells regarded as possible sources of N hormone, two kinds of neurosecretory cells (α and β neurosecretory cells) were described, which are located in areas of the nervous system more or less distant from the area of distribution of the γ cells. These show a cytoplasmic secretory activity with particulate elements as their ultimate products. The physiological significance of their respective secretions is still unknown.

6. The sinus gland is innervated by a cluster of giant β neurosecretory cells of the medulla terminalis. Figures show a distal flow of secretory granules from the cells along the course of the sinus-gland nerve, a fact which is indicative of an interesting type of chemical transmission resembling those reported to occur in the innervating systems of the vertebrate hypophysis and of the insect cardiacum-allatum complex.

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CHLORIDE ION REGULATION AND OXYGEN CONSUMPTION IN THE CRAB OCYPODE ALBICANS (BOSQ)

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There are a number of species of brachyuran crabs which are terrestrial or semi-terrestrial, in contrast to the majority which are strictly aquatic. One of the relatively successful terrestrial crabs of the Atlantic Coast beaches is the "ghost" crab, *Ocypode albicans* (synonyms *O. arenaria,* Say and *O. quadrata,* Fab.). Ocypode burrows in sandy beaches, near or above the high tide level. On the Delaware beaches, where we collected it, the crab is nocturnal and spends the day in its burrow. At night it runs about the beach hunting food, and during this time it may go into the surf briefly. When alarmed, the crab runs for its burrow in preference to the sea. The incoming tide sometimes traps it in its burrow and forms a sand plug in the opening. When the tide recedes, the crab removes this plug and the burrow is useful again. So Ocypode is a crab which successfully lives in a terrestrial habitat, although this habitat borders very closely upon the sea. The anatomical adaptations of this crab have been described by Cowles (1908) and Pearse (1928).

Some brachyurans show a considerable degree of the regulatory ability which results in the maintenance of a steady internal osmotic state regardless of the fluctuations in the external environment. The degree of osmotic regulation of several species of crabs has been described by Jones (1941). He found a direct correlation between the degree to which the regulatory ability was developed and the success the crabs had shown in meeting the conditions of terrestrial life, or life where drying was a constant environmental factor. In his series Uca was the most nearly homoiosmotic. If this correlation can be projected to Ocypode, which belongs to the same family, but is more terrestrial in its habits, Ocypode also should show a high degree of osmotic regulation.

The specific mechanism of water and ion control in Ocypode has not been described. However, it is clear from the literature (Krogh, 1939) that the principle structures concerned in the crustacea are the antennal glands (kidneys) and the gills. The regulatory mechanism certainly operates at these barriers between the internal and external environments; in Ocypode it is not probable that the integument is very permeable to water and electrolytes. The regulation of water and ion movement through a membrane against a gradient is not a passive activity, but requires an output of energy during the process. Schlieper (1929) proposed the theory that homoiosmotic forms respire more rapidly in dilute than in normal sea water, while this is not the case with the poikilosmotic forms, and he suggested that energy expended by the former in resisting osmotic inflow of water is derived from some oxidative mechanism. On this basis it would be expected that any work done by a membrane upon exposure to a changed external environment would be reflected in the oxygen consumption of the animal.

The investigations reported here were concerned with the following aspects of the problem of regulation: first, the degree to which Ocypode carries on chloride ion regulation; second, the role of the antennal gland in chloride ion regulation; and third, a measurement of the oxygen consumption during the early stages of this regulation.

METHODS

Crabs were collected at Rehoboth Beach, Delaware, placed in separate containers and brought back to the laboratory within a few hours of capture. They were kept in individual battery jars in which there was enough beach sand to allow burrow digging. This sand was kept moist but not soaking wet. The jars were covered to keep out direct light, and to prevent the escape of the animals. The crabs were fed all the fresh fish they would eat each night and allowed to swim in sea water for about five minutes. The once-a-day feeding and bathing routine paralleled natural conditions as we observed them and permitted us to keep the crabs in good condition for ten days or longer. Crabs had been in the laboratory for at least twenty-four hours before any work was done with them.

The degree of regulation of a single ion, the chloride ion, was studied. It is not presumed that a study of this ion alone gives a complete picture of the shifts in the internal environment. However it may be used as an indicator of the direction and degree of internal regulation.

The chloride ion concentration in the blood from individual crabs was determined. Blood was drawn from the sinus within the proximal joint of one of the legs, using a No. 27 needle fitted on a clean, dry one-quarter cubic centimeter tuberculin syringe. The usual amount of blood drawn at any time was about 100 cubic millimeters. No anticoagulant was used. It was found that quick smooth handling of the blood could effect the transfer from crab to analytical tube before clotting commenced. After the blood sample was drawn, the needle was removed from the syringe, a twenty cubic millimeter Sahli type haemoglobin pipette held to the nozzle of the syringe, and blood measured into the pipette to the calibration mark. The sample was blown into a small test tube, and the pipette rinsed into the test tube with distilled water. Samples were analyzed in duplicate, using a modification of the Volhard method. Silver nitrate was made up to 0.2906 per cent in nitric acid with ferric ammonium sulphate added as an indicator. An excess of this reagent, usually one cubic centimeter, was added to the blood sample in the small test tube. Potassium sulfocyanate solution, made up to one-half the strength of the silver reagent, was used to titrate the excess silver. Reagents were added with the syringe burette described by Scholander (1947).

Urine samples were collected by applying suction to the opercula which close the external openings of the antennal glands of Ocypode. A small vial, capacity approximately two cubic centimeters, was fitted with a rubber stopper through which passed two No. 18 syringe needles. One of these was connected by pressure tubing to a vacuum line. The cuff of the other was removed, and replaced by a short length of soft rubber tubing which could be fitted closely over the operculum. Urine collected in this way was analyzed for chloride ion content by the method used for blood.

The chloride ion present in sea water used was determined by the same method. Sea water taken at the beach from which the crabs were collected was brought back to the laboratory, filtered and used in the experiments. It contained 480 millimoles of chloride per liter, which conforms to the figure for normal sea water used by Cole (1940). The value is lower than that determined for some open sea samples. This fact may be accounted for considering that Rehoboth Beach, Delaware, is but a few miles south of the mouth of the Delaware River. Distilled water was used to obtain dilutions amounting to 360, 240, and 120 millimoles of chloride per liter. Evaporation in shallow pans under a fan at room temperature resulted in concentrations of 600 and 720 millimoles of chloride per liter. In addition to the sea water concentrations, some crabs were put into distilled water, which is recorded as 0 millimoles of chloride per liter. Others were not put into any of the experimental solutions but remained on damp sand and are recorded as "dry."

The oxygen consumption of the crabs was measured in a respirometer of the direct reading volumetric type, similar in principle to the micro-respirometer of Scholander (1942). The apparatus is shown in Figure 1. The animal chamber (1) was constructed from a finger bowl, the rim of which was ground flat and smooth. An alkali cup (2) made by drilling out a piece of one inch plexiglass rod was fastened to the bottom of the bowl with DeKhotinsky cement. A shield of plastic screen (Lumenite) placed around the cup prevented the animal from getting into the alkali. The lid of the animal chamber (3) was a disc of quarter-inch plexiglass, recessed at the periphery to close on the ground rim of the finger bowl. Three holes were drilled in the disc and fitted with rubber stoppers as shown in the figure. A three-inch length of glass tubing through one of the peripheral stoppers served as a by-pass through which fluids were added to the chamber during an experiment. A "T" tube through the central stopper had a short arm to which a ten cubic centimeter syringe (4) was attached. A small "U" tube passing through the third stopper was connected to the manometer.

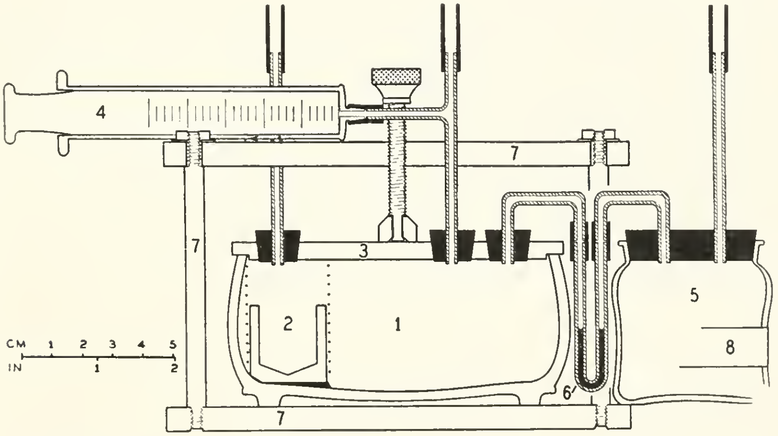

FIGURE 1. A volumetric respirometer (for explanation see text).

The thermobarometer (5) was made from a wide-mouth specimen jar fitted with a two-hole rubber stopper. A by-pass tube through one hole opened the chamber to the outside air. A small "U" tube connected the thermobarometer to the manometer. The manometer (6) was a tight "U" about two inches long. All glass connections in the unit were made from two millimeter bore glass tubing. Rubber junctions and extensions on the by-pass tube were made from number fourteen rubber catheter. Light weight hose clamps (not shown) were used to close the extensions.

The lid of the animal chamber was closed onto the finger bowl by a stirrup clamp (7). A four by six inch plastic platform was drilled to take two uprights made from quarter inch brass rod. The upper cross member of the clamp was a strip of plastic drilled to pass the threads of the uprights. The center point of this strip was drilled and tapped to take the thread of a thumb screw carrying a knurled knob on one end and a freely turning caster on the end bearing on the lid. The finger bowl rested on the platform. The syringe was fastened to the cross member by heavy rubber bands. The thermobarometer was fastened to one of the uprights with a half inch metal strap (8).

In use the interior of the finger bowl was moistened with water to saturate the atmosphere, and the rim was coated with heavy stopcock grease. Two cubic centimeters of 10 per cent KOH were placed in the alkali cup with a strip of folded filter paper to increase surface for absorption. The interior of the thermobarometer was moistened with water through a by-pass tube. A drop of paraffin oil spread on the plunger made the syringe airtight. The crab was weighed and placed in the finger bowl, which was then set on the platform of the stirrup clamp. The lid was put in position and tightened by means of the thumb screw. The manometer was filled to a height of three-quarters of an inch with 0.1 per cent aqueous tergitol solution and joined to the "U" tubes of the two chambers. A rider made of white plastic sheet was found useful in reading the menisci of the manometer. With all outlets open, the unit was placed in the water bath with the tops of the tubes out of water. The by-pass of the animal chamber was closed. The syringe was charged by attaching an oxygen-filled bladder to the free end of the "T" tube, pulling out the plunger of the syringe, then closing the upper end of the "T" to prevent loss of oxygen from the system. The by-pass of the thermobarometer remained open during the equilibration period of twenty minutes. The syringe was constantly adjusted inward to keep the menisci of the manometer level. At the end of the equilibration period the thermobarometer by-pass was closed, and the initial reading of the syringe recorded. During the course of the experiment oxygen consumed was replaced from the syringe, and the syringe readings gave a direct measure of the oxygen consumption of the animal. The respirometry was done during the summer at a room temperature of 26° C.

The oxygen consumption of the crabs which are described as "dry" in the respiration results was measured in this way for a period of an hour and a half. Regassing the syringe during this time was done without removing the apparatus from the water bath, by attaching a bladder to the "T" tube. Added oxygen was in thermal equilibrium with the water bath. Crabs which were tested for response to a new environment were treated in the following way. The oxygen consumption was determined for thirty minutes, with the crabs in a moist chamber as described. At the end of thirty minutes, the taps were opened, and one hundred cubic centimeters of water of a measured salinity were introduced into the respirometer chamber through the by-pass tube. This water had been well shaken with air, and had been allowed to equilibrate to the temperature of the water bath. The taps were closed and oxygen consumption of the same crab measured for a full hour. In this way, the sequence of oxygen consumption in air and in environmental fluids of various salinities could be measured.

Blank runs, with the animal chamber moist and with the introduction of water were made following the exact experimental procedure except that no animal was used. The results showed no measurable drift for two hours following equilibration.

RESULTS

Ocypode spends most of its time out of water, either in burrows or running on the sand. The blood chlorides of crabs from sand were determined as a normal value. The mean of sixty-four "dry" determinations was 378 mM Cl/L, with a standard deviation of 24.27. This indicates that two-thirds of the data falls between the values of 354 and 402 mM Cl/L, a spread of 12.7 per cent. The mean is well below the figure for normal sea water, which was 480 mM Cl/L, being much nearer 75 per cent sea water, or 360 mM Cl/L. In view of the spread of the "dry" determinations, it was thought more valuable to compare the reaction of a crab to a new environment with its own "dry" chloride level rather than with a mean of that group or of a control group. The experimental procedure was, then, to determine the chloride of a crab on sand, expose the crab to the experimental sea water, and determine the blood chloride again at specific time intervals. The ratios of the experimental to dry values were calculated. The mean of the ratios for three to six individuals in each environmental situation after one, three, and twenty-four hours are plotted in Figure 2, with the standard deviations in each case represented by a vertical line. One group of crabs remained on sand; the blood chloride was determined for the same time intervals, and treated in the same way. The ratios are recorded in Figure 2 as "dry."

There are no significant changes in the blood chloride levels of individual animals exposed to the various environments for one or three hours. The blood chloride of Ocypode remains steady for a period of twenty-four hours in air, and under the conditions of exposure to sea water diluted to 120 and 360 mM Cl/L and evaporated sea water containing 600 mM Cl/L. There is some difference in the ability of individual crabs to maintain a steady internal chloride level, but the fluctuations observed are not statistically significant. When the crabs are exposed to distilled water or to water containing 720 mM Cl/L for twenty-four hours, the internal chloride does not remain steady, but tends to fall in distilled water and to rise in the high concentration of sea water. Within twenty-four hours there is a significant change in the internal chloride in these extreme ranges although these values do not yet equal those of the external environment. It was found difficult to carry the crabs beyond this time, or to restore them in normal sea water, although they survived three days or longer in all the other environments.

Urine was collected from crabs on sand and from crabs which had been in experimental solutions. An attempt was made to collect urine at one hour, three hour and twenty-four periods, but it was found that the shorter intervals were unsatisfactory. No attempt was made to determine the volume of urine. Ocypode often ejects the urine when handled, and many samples were lost in this way. Crabs from sand were most often lacking in available urine but it was usually obtained after they had been in any of the solutions for twenty-four hours. The urine chlorides of crabs on sand were determined. The mean of eleven "dry" determinations was 455 mM Cl/L, with a standard deviation of 46. The mean value falls above that for blood, and below that for normal sea water. The results of urine chloride determinations at the twenty-four hour period were treated in the same way as those of the blood chlorides, and are plotted in Figure 3. The mean ratio of urine chloride of three crabs in each environment is plotted, and these points are connected by a solid line. The standard deviation in each case is indicated by the vertical line. The urine chloride ion concentration varies in the same direction as the concentration of the environment, being low in dilute solutions, high in concentrated solutions. There is a wider deviation in the results in the extremely dilute and extremely concentrated environments than there is towards the more nearly isotonic environments.

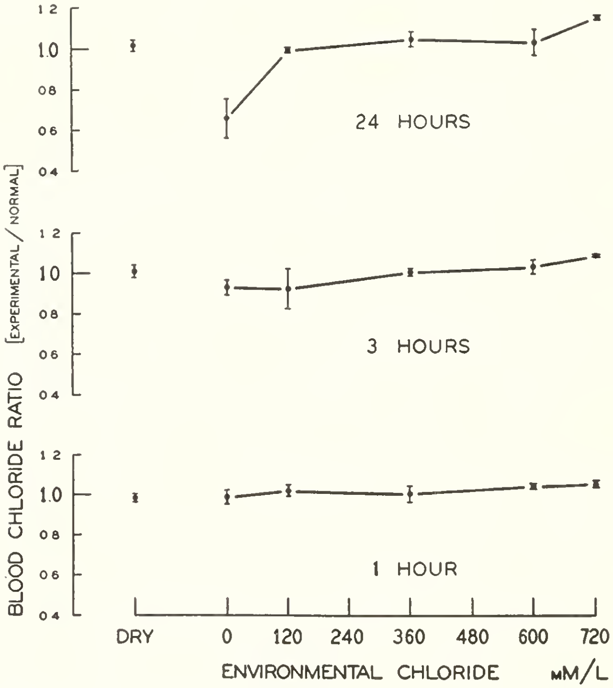

FIGURE 2. Relationship between blood chloride and environmental chloride concentrations.

To determine the effect of the regulatory work on the oxygen consumption, the respiration of crabs from sand, and of crabs in sea water of a range of salinities was measured. The oxygen consumption of six individuals from sand was determined on each of three consecutive days. The mean and the standard deviation were calculated for each of the six individuals, on the basis of data from three consecutive daily determinations. The six means and standard deviations so determined were averaged to give the average mean and average standard deviation of six individuals. The mean oxygen consumption was 0.139 cubic centimeters of oxygen per gram fresh weight per hour, with a standard deviation of 0.019. This indicates that about two-thirds of the data fall in the range 0.120 to 0.158 cubic centimeters of oxygen per gram per hour. This treatment gives a more representative picture than would (1) the standard deviation of all eighteen determinations, which would deny that there were six sets of repeat determinations; or (2) the standard deviation of the six means of three determinations each, which would mask the fluctuations within a series of determinations on each crab. It is apparent that the conventional use of a volume of data as the "normal" or control is impossible in the face of such wide deviations. It was decided, therefore, to compare the response of a single crab to a change in the external environment with the dry oxygen consumption of the same crab. This conformed to the treatment of the chloride data.

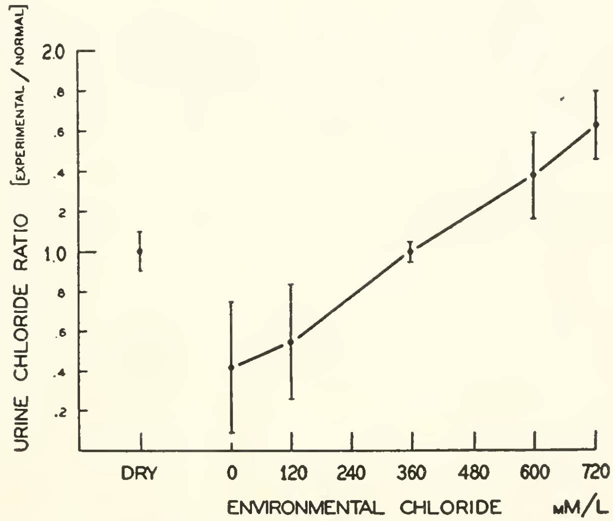

FIGURE 3. Relationship between urine chloride and environmental chloride concentrations after twenty-four hours.

In the respiration measurements, the oxygen consumption of the crabs was determined the first thirty minutes with no standing water in the moist chamber, hence the animals could be termed "dry". This thirty minute period is designated "A". The oxygen consumption was determined for one hour after the experimental sea water was added to the respirometer. The first thirty minutes after the addition is designated "B", and the second thirty minutes "C". It could be predicted that if the oxygen consumption rate did not change, but a steady rate was maintained during the entire ninety minutes, period "B" would equal "A", and periods "B" plus "C" would equal twice "A". A graph of this steady state would be a straight line.

TABLE I

Environment in mM Cl/L	"B"/"A"	"B" + "C"/"A"
0	1.50	2.53
120	1.54	2.59
240	1.58	2.73
360	1.15	2.17
480	1.41	2.69
600	1.38	2.57
720	1.35	2.61
"Dry" (normal)	0.95	1.84
predicted ratio	1.00	2.00

The data on oxygen consumption were calculated as ratios of the period "B" to period "A", and of period "B" plus "C" to period "A". Six individuals were tested on three consecutive days for each of the environmental situations, and the data calculated as outlined above for the normal rate of oxygen consumption. Table I presents the mean of the ratios so obtained. In Figure 4 the data are presented graphically, with the mean of six individuals plotted as the central point, and the mean standard deviation indicated by a vertical line for each set of determinations.

The rate of oxygen consumption was consistently higher in water, regardless of the concentration, than under the dry circumstance. This cannot be attributed

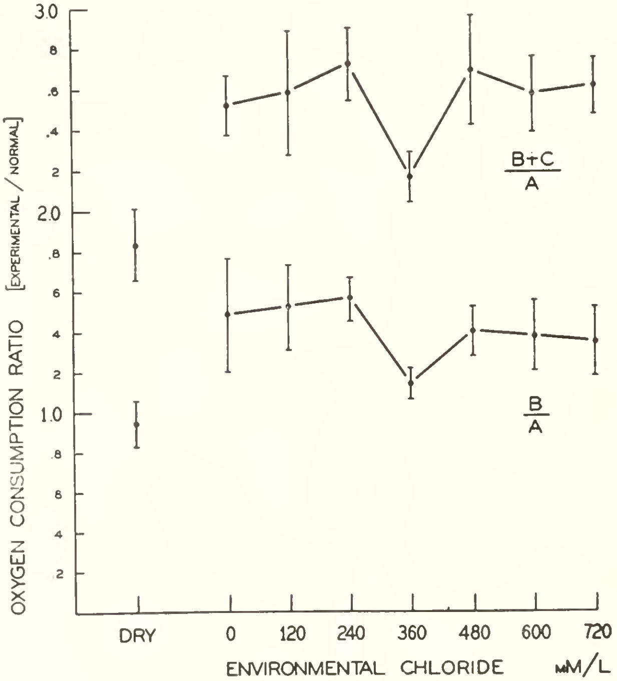

FIGURE 4. Oxygen consumption related to environmental chloride.

to diffusion of gas into the introduced water or oxygen consumption by organisms in the water. Check runs complete except for the crab failed consistently to give discernible evidence of any but a steady state of the respirometer. The rate of oxygen consumption in water containing 360 millimoles of chloride per liter was significantly less than the rate in any other salinity. This concentration most closely approximated the normal internal chloride concentration of the crab, which was determined as 378 millimoles of chloride per liter. In concentrations of sea water greater than or less than this nearly isotonic concentration, the rate of oxygen consumption increased significantly. The increase in dilute solutions was greater than in hypertonic solutions. There was no evidence of progressive increase as the extremes of salinities were approached. There was, however, a wider fluctuation in individual results, particularly in the dilute series.

Discussion

Ocypode maintains a steady chloride ion concentration in the blood in air and against a wide range of external chloride concentrations. The mean blood concentration is 378 millimoles of chloride per liter, and is held steady against external concentrations ranging from 120 to 600 millimoles of chloride per liter. When the crab is exposed to external concentrations of 0 and 720 millimoles of chloride per liter the steady state is not maintained and a drift towards the lower or higher value is observed. At the end of twenty-four hours in these two extremes, the chloride ion concentration in the blood falls as much as 40 per cent or gains as much as 15 per cent over the normal level. No observations were obtainable on crabs which had been exposed to the environmental extremes for several days. It was very difficult to keep them alive beyond the twenty-four hour period, especially in distilled water. The decreasing viability probably is not due to the shift in chloride ion concentration alone, but is indicative of a breakdown in the mechanisms regulating water and ion transport.

The ability of Ocypode to regulate the chloride ion concentration of the blood is in agreement with the observed ability of the more terrestrial crabs, Uca, in particular, to approach a homoiosmotic condition. Jones (1941) has shown that Uca regulates against both hypotonic and hypertonic environments. The fact that the mean value of blood chloride for Ocypode is well below the figure for sea water, which was 480 millimoles of chloride per liter at the site where the crabs were taken, indicates that the crabs which made the transition from sea to land possess the mechanism fundamental also to those which move from sea to brackish water. According to the hypothesis proposed by Beadle and Cragg (1940), a contributing factor to successful transition from sea to brackish or fresh water is the maintenance of a chloride ion concentration in the blood somewhat lower than that of sea water, hence, presumably easier for the animal to maintain against the brackish water gradient. Other members of the family Ocypodidae, Uca for example, invade brackish water as well as spending much time in air. The lower internal chloride concentration of Ocypode is obviously easier to maintain in the terrestrial habitat wherein food and infrequent contact with sea water are the only sources of chloride ions.

The maintenance of a steady internal chloride ion concentration is accomplished by regulation of water intake and loss, and salt intake and loss. The data we have collected on urine chloride concentrations indicate that the antennal gland of Ocypode functions in chloride ion control. If the chloride ion content of blood and urine from crabs on sand, the "dry" or "normal" values, are employed with the ratios shown in Figures 2 and 3, and the representative levels of blood and urine chloride under the imposed environmental conditions are calculated, a graph such as Figure 5 may be made. Calculated levels for urine chloride are indicated by a solid line, for blood by a broken line, and for the environmental fluid by a dotted line. The line representing the environmental fluid crosses the line for blood chloride at the isotonic level, about 380 millimoles of chloride per liter. In the situation of the chloride ion concentration in the external environment being equal to that in the internal environment, no accumulation of chloride in the urine would be expected. However, as the data indicate, chloride ion is present in the urine in excess at the isotonic point. This is probably due to the re-absorption of water by the antennal gland. Supporting evidence for this conclusion may be drawn from two observations: first, the urine chloride ion content when the crab is in a medium containing 360 mM Cl/L is not significantly different from the urine chloride concentration of crabs on sand. Water saving by re-absorption is important for a terrestrial animal, and it would appear to be the activity reflected here. Second, the oxygen consumption of crabs in a medium containing 360 mM Cl/L is the lowest of all the rates of

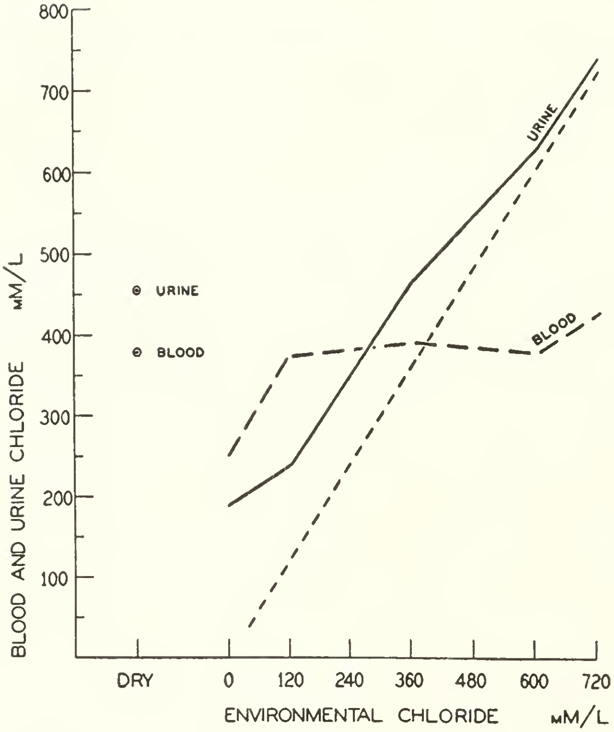

FIGURE 5. Calculated blood and urine chloride concentrations related to environmental chloride.

crabs in fluid environments, indicating a minimum of regulatory work being done.

The chloride ion concentration in the urine varies in the same direction as that in the environment. However, the urine chloride concentration is equal to the blood concentration only when the crab is in an envionment containing about 270 mM Cl/L. In sea water containing more chloride than this value, the urine tends to contain more chloride ion than the blood; in more dilute media the concentration of chloride ion in the urine is less than in the blood. Here is indication that the antennal gland is active in the control of the chloride ion concentration in the internal medium, either actively secreting chloride or re-absorbing water, over a considerable range of external environmental salinity. This is an expected activity when the chloride ion concentration in the environment is higher than that in the blood. Further examination of the data shows that in very dilute solutions the antennal gland wastes chloride, producing a urine that has a high chloride ion content as compared with the external environment. The fact that a high urine chloride loss persists in distilled water, despite the fact that the blood chloride level is falling, may be interpreted to mean that the antennal gland does not have a critical threshold for the chloride ion.

From Figure 5 it may be seen that at the 270 mM Cl/L level in the external environment, blood and urine chloride concentrations are equal. Animals in this range of environmental chloride survived for several days and maintained a normal and steady blood chloride level. It is clear that at this point proportionately more chloride than water must be obtained from the external environment. The fact that the blood chloride level is maintained steady in hypotonic environments in spite of an overactive excretion of chloride by the antennal gland is further evidence that some chloride-absorbing mechanism is at work. It is assumed that the mechanism here is selective absorption of chloride ion by the gills. Webb (1940) has shown that the gills of Carcinus do actively absorb chloride ion from the external medium. He has further shown that there seems to be no reason to assume an irreciprocal permeability of the gut and integument. It might be pointed out that the integument of Ocypode does not appear to permit evaporation of water when the animal is on land, and it is assumed that it would not permit great exchanges of salt and water when the crab was immersed. We have not yet completed studies as to the possibility of specific tissues being reservoirs of internal salts and water.

It is seen from the data on oxygen consumption of crabs from sand and from environments of different salinities that work is being done to maintain the internal chloride ion concentration, and, it may be projected, the internal osmotic state. The membranes involved, gill and antennal gland, although affording ample surface for the exchange of materials, do not make up a large part of the mass of the animal. In order to determine the metabolic reaction to changing environments it is necessary to use an intact animal, and to measure a change dependent on the activity of a very small per cent of the weight of the animal. It was felt that with the combination of measuring a small overall change and the inherent variation between individuals, statistical treatment of the data would prevent overlooking pertinent relationships.

In order to relate the data obtained on oxygen consumption to the observed regulation of the internal chloride ion concentration, the ratios of the first experimental period to the control period ("B"/"A") are represented in Figure 6 in block form. If the oxygen consumption in air (solid segment) is assumed to represent the normal or control value, and if this amount is then removed from the values for oxygen consumption in water, the increment in water must be due to work, either of a mechanical or osmotic nature. The increment is least, about 21 per cent, in an external environment of 360 mM Cl/L. From Figure 5 it is seen that at this concentration the blood and external chloride concentrations are nearly equal. As suggested earlier, at this environmental level, the gill is probably doing little or no osmotic work, since no gradient exists between internal and external environments. On the basis of the urine chloride data, the antennal gland work at this level is no more than is being done by the "dry" crab on sand. Therefore, most, if not all of the increment in oxygen consumption at 360 mM Cl/L is due to mechanical or muscular activity. In view of the greater mass and viscosity of water it is reasonable to assume that water bailing requires more energy than air ventilation. Ocypode has reduced gills and branchial tufts according to Pearse (1928). In air the diffusion of oxygen through these respiratory surfaces is sufficient for the requirements of the animal, probably with no more accessory ventilation than is accomplished during leg movements. In the respirometers the crabs showed a definite thigmotactic response and were quiet during the time of the experiment. When Ocypode is placed in water, a scaphognathite attached to the second maxilla sets up a rapid beat which serves to force water from the posterior branchial chamber opening between the bases of the third and fourth legs through the branchial chamber and out at the mouth region. Two "gill rakers" attached to the first and third maxillipeds also beat rapidly when the crab is in water. This constant muscular activity which is not present in crabs in air may account for the increase in oxygen consumption in environmental fluid containing 360 mM Cl/L.

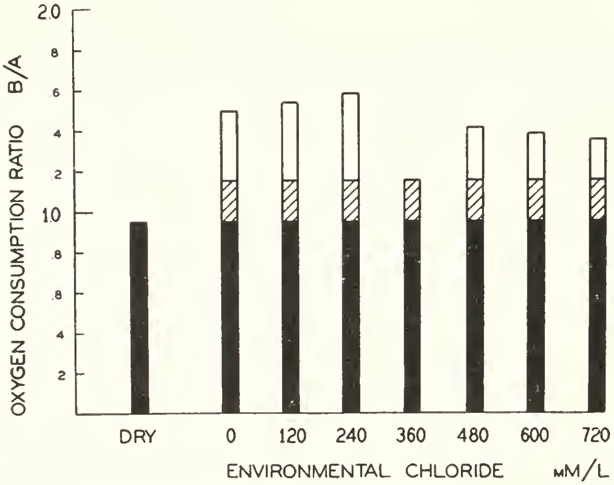

FIGURE 6. Oxygen consumption related to environmental chloride to show partition of work between air and water ventilation and between water with and without chloride gradient.

A possibility which merits further investigation is that some of the increase noted when the animals were placed in water may be due to the payment of an oxygen debt incurred as a result of drying of a significant portion of the respiratory surface, or as a result of inadequate ventilation in air when the animal is quiet. In an environment containing 360 mM Cl/L, the ratio of respiration in the first experimental period to the control period is 21 per cent higher than the same ratio of crabs "dry" throughout the experiment. The ratio for the second experimental period is only 14 per cent higher than the same corresponding ratios for "drys." Measurement of the respiration was not continued past this point. Not enough is known of the respiration pattern of Ocypode to make a definite statement concerning the possibility that these increments indicate payment of oxygen debt. However, two observations may be considered here: (1) crabs on moist sand ten days are just as active and maintain the activity as well as crabs exposed to periodic wetting, which would indicate no cumulative oxygen debt; and (2) the pattern of decrease in the second period does not change with increasing time of exposure to air. The possibility of early saturation of the oxygen debt capacity complicates this latter observation. It may be speculated that if there is an oxygen debt present in Ocypode in air, this represents a phase of terrestrial adaptation which has not yet become perfected in this species.

The increase in oxygen consumption in 360 mM Cl/L as compared with that in air is represented in Figure 6 by the cross hatched segment. If this portion, plus that corresponding to the respiration in air, is then removed from the total block for each environment, the remaining clear segment may be assumed to represent the osmotic work done in maintaining a steady internal environment against a gradient. In hypotonic solutions the increase attributable to this regulatory work is of the order of 34 per cent, decreasing slightly in the more dilute environments. This increase represents the sum of the work of two regulating mechanisms. The gill membrane is working to absorb chloride ion from the environment, and is allowing water to pass into the crab, while the antennal gland is bailing water out again. In the more dilute environments the antennal gland appears to be inefficient. In Figure 5 it is seen that the amount of chloride excreted is in excess of what would be predicted on a straight clearance basis, which would indicate that the antennal gland is failing to re-absorb chloride ion or is not bailing the water necessary to maintain the balance. Webb (1940) has suggested that in very dilute solutions the gill of Carcinus becomes less permeable to water. The data presented here seem to be in accord with this suggestion. The gill continues to absorb chloride ion from the environment, while allowing less water in proportion to pass the barrier; the antennal gland then clears less water, with less energy expenditure than would be expected. The two mechanisms are able to maintain an internal chloride ion level in solutions containing 120 millimoles of chloride per liter or more. No points intermediate between this dilution and 0 were tested, so that the exact limit to which the regulation is maintained is not known.

In solutions containing more than 360 mM Cl/L there is an increase in the rate of respiration of the order of 20 per cent. In hypertonic solutions the gill ceases the selective absorption of chloride ion from the environment, and as far as we know, has no chloride-secreting mechanism. However, chloride ion and water pass through the gill membrane in accordance with the Donnan equilibrium. The antennal gland is the chief mechanism of regulation in hypertonic environments, excreting chloride ion or saving water, or both. The antennal gland working alone does not require as much oxygen as gill and antennal gland together do in hypotonic solutions. In environments of high concentration, 720 mM Cl/L, the ability of the antennal gland reaches a limit and cannot keep up with the incoming chloride ion. Hence, the blood chloride ion level rises. The upper limit on antennal gland ability is also shown by the fact that the oxygen consumption does not progressively increase with increase in environmental chloride. The slightly lower oxygen consumption in the greatest concentration may be the result of metabolic depression caused by the concentration of ions other than chloride.

With the hypothesis of Schlieper (1929) in mind, inspection of the oxygen consumption rate across the spectrum of osmotic insult would indicate the point at which the external and internal environments approached isotonicity. The elevation of the oxygen consumption rates on either side of this isotonic point may be accounted for, as indicated above, by the energy output of gill and antennal gland, each in a twofold role of chloride ion capture and clearance.

Further examination of the respiration and urine chloride data indicates that the deviation from the mean is much wider at the environmental extremes, than towards the isotonic level. It may be postulated that here is shown a range of response of single factor magnitude which might form a basis for selection. In the past, the antennal gland function, especially on the hypertonic side and in air, may have been an important factor in the selection of crabs as they passed from water to the terrestrial habitat.

SUMMARY

1. *Ocypode albicans,* a brachyuran crab with a terrestrial habitat, is able to regulate the chloride ion content of the blood in the dry habitat and in sea water of salinities ranging from 120 to 600 millimoles of chloride per liter. The mean value of the chloride ion concentration in the blood is 378 millimoles of chloride per liter. This value is lower than that of sea water at the site the crabs were collected (480). It is suggested that the lower concentration of chloride is easier for the terrestrial animal to maintain in an environment where chloride is available only from food or from occasional contact with the sea.

2. In sea water containing less than 120, or more than 600 millimoles of chloride per liter, the internal concentration is not maintained, but tends to fall in dilute solutions, rise in the more concentrated ones. This internal shift is significant in a period of twenty-four hours, and proves fatal if continued for an additional twenty-four hours. The antennal gland functions in the regulation of the internal chloride ion concentration. It re-absorbs water when the crab is in air and in hypertonic solutions. It excretes water when the crab is in hypotonic solutions, and may excrete chloride also. The evidence points to another organ functioning in the reverse way; this is assumed to be the gill membrane.

3. The oxygen consumption of Ocypode in air is of the order of 0.139 cubic centimeters of oxygen per gram fresh weight per hour. In water the crab uses a gill bailer and gill rakers constantly, and the respiration rate is elevated. The increase is least when the crab is in an environment in which the chloride ion concentration equals that of the blood. In hypotonic and hypertonic environments the oxygen consumption is further increased. This is caused by the increased activity of the ion-regulating membranes of the antennal glands and gills.

4. Comparison of individual responses to the different environments indicates a difference in individual ability to meet the stress. The differences are more widely marked in environments most different from the internal medium. It is suggested that this is a measure of the variation of a physiological mechanism which was probably important in the evolution of the crab from the aquatic to the terrestrial habitat.

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PHOSPHATE UTILIZATION BY DIATOMS[1]

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Routine measurements of inorganic phosphorus concentration are frequently made on standard oceanographic stations to ascertain the potential productivity of marine waters. In quantitative, ecological studies on diatom populations in the Atlantic Ocean, Riley, Stommel and Bumpus (1949) have utilized the phosphate concentration as a nutrient index. Their equation of productivity depends upon the experimental results of Ketchum (1939), who found that phosphate concentrations below 0.55 microgram-atoms per liter limited the growth of *Nitzschia closterium.*

Riley, Stommel and Bumpus (1949) further checked the validity of this index in their photosynthetic rate equation by calculating the phosphate requirements of a growing plankton population, assuming that 100 carbon atoms to one atom of phosphorus are needed for normal metabolism. However, in studies on the fresh water diatom *Asterionella formosa,* Lund (1950) found that the phosphate content of the plant varied by a factor of seventy in both natural and cultural populations. He stated that the cells are able to store phosphorus in excess of their immediate requirements even at very low levels of phosphate concentration.

The data on the amount of phosphate contained in marine phytoplankton, as found by Ketchum and Redfield (1949), show considerable variation, making the determination of phosphate requirement difficult and uncertain. The use of phosphate as a parameter in the computation of productivity would appear to be premature therefore, until such time as definitive values of the phosphate requirement shall have been established. Even then the total mineral requirements relative to one another must be known and their seasonal and geographic abundances must be shown to vary with biological activity. The use of an arbitrary and easily measured nutrient index for correlation with diatom abundances seems to be unwarranted.

The release of phosphate by marine plankton is an important factor in ecological investigations. Cooper (1935) found that one-fourth to one-half of the phosphate added as phytoplankton in sea water was set free to the environment as inorganic phosphate after one month. Seiwell and Seiwell (1938) reported similar results.

Because of the scarcity of experimental data on the uptake of phosphate and its subsequent release from diatoms, controlled experiments using radioactive phosphate as a tracer were devised to follow the path of phosphate quantitatively from the marine environment to the plant as a function of time. This present study initiates a series of investigations which will attempt to determine by modern radiochemical techniques the minimal quantities of mineral nutrients needed for optimal growth and multiplication of a diatom species in culture.

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[1] Contribution from the Scripps Institution of Oceanography, New Series No. 547.

LABORATORY TECHNIQUES

Stock cultures

Single cells or colonies of cells were isolated from inshore water samples taken at the pier of the Scripps Institution of Oceanography, La Jolla, California. Isolated cells of a given species were washed by passing through three drops of sterile nutrient sea water medium, and were then placed in 10 ml. of medium in a test tube. When the diatom population had reached approximately 1000 cells per ml., a one-ml. aliquot was transferred to a cotton-stoppered 250-ml. Erlenmeyer flask containing 100 ml. of a sterile nutrient sea water medium, as described below. Cultures were maintained by transferring one ml. of culture to a flask of fresh medium at two-week intervals. The original cells of the *Asterionella japonica* culture used in the early part of the work had been isolated from the plankton sample in the fall of 1948; the initial cells of the *Asterionella japonica* later utilized were isolated from the plankton samples in the winter of 1950.

Preparation of the nutrient sea water medium

A basic requirement in the radiochemical assay of the partition of phosphate between the nutrient medium and the diatoms is that the total phosphate be contained only within these two fractions. Thus, any phosphate precipitation during the preparation of the enriched sea water or during the course of the experiments invalidates the measurements. Microscopic examination of culture waters prepared in the normal way revealed particulate matter which was found by chemical analysis to be iron phosphate. This solid phase was able to adsorb and to contain a considerable portion of the phosphate. Since the cells often clung to, or were entrapped, or covered by these masses, these precipitates tended to obscure the diatom cells during visual counting, making the counting more difficult. Therefore, the sea water collected for use in the culture medium was subjected to special treatment before and during enrichment to prevent any formation of particulate matter.

The sea water used in the preparation of the medium was collected in glass containers, approximately 14 liters at a time, from the pier. It was filtered through either S & S No. 489 Blue Ribbon or Whatman No. 42 paper before enrichment with nutrients.

The medium used was a modification of Allen and Nelson's (1910) culture solution (Table I). The sea water and the nutrients were sterilized in separate containers. The salts were sterilized in two portions: one, a combination of the KNO_3, $MnCl_2$, and Na_2HAsO_4; the other, the phosphate made up to 100 ml. of

TABLE I

Culture medium nutrients

Solution		Amount of solution added per liter
M/10	KNO_3	20 ml.
M/100	$MnCl_2$	0.1 ml.
M/100	$CoCl_2$	0.1 ml.
M/100	Na_2HAsO_4	0.1 ml.
3M/1000	K_2HPO_4	Varied through investigation
M/10	Ferric citrate	0.1 ml.

solution with distilled water. The sea water was also sterilized in two portions of three liters each in 4-liter reagent bottles. Sterilization was achieved in the autoclave at 120° C. and 18 pounds pressure. Exposure times were 10 minutes for the salts and 25 minutes for the sea water. After cooling, the water and salt solutions were combined in a sterile 9-liter serum bottle, the salt solution entering through a separatory funnel as the sea water simultaneously passed through glass tubing to the bottom of the bottle. The KNO_3 combination was added with the first three liters of sea water, and the phosphate with the second portion of the sea water. The iron citrate solution was added directly to the culture bottles before diatom inoculation.

Bio-assay

Water samples sometimes failed to produce growth in spite of enrichment. To avoid cultural failures, all of the water collected was first assayed. After preparation of the enriched water, a sample was drawn and placed in several stock culture flasks and then inoculated. An initial count was made and the cultures re-counted twice at weekly intervals. Not infrequently these preliminary assays showed that, in spite of enrichment, the water was refractory to diatom growth. Such water was never used in the radiochemical experiments.

Radiochemical culture techniques

Seven hundred ml. of the enriched sea water was added to the one-liter wide-mouthed culture flasks (Fig. 1), which were stoppered with cotton plugs. The water within the culture bottles was stirred by means of a floating magnetic stirrer made from a 50-ml. round bottom boiling flask. In initial experiments in which the magnetic stirring rod was allowed to rest on the bottom of the culture bottles, the diatoms were destroyed. The solutions were stirred for a period of one minute out of every fifteen minutes with a Haydon Repeat Cycle Timer. It was found that at this stirring rate all of the diatoms received adequate light for normal growth. Continuous stirring resulted in some diatoms clinging to the walls of the glass vessel.

Six such flasks were kept in a constant temperature room at 18° ± 2° C. Five of the flasks contained diatoms while the sixth flask was used as a radiochemical control. The six culture flasks received an equal illumination from a 48-inch, forty-watt fluorescent lamp. The light intensity, as measured with a Weston Photronic Foot Candle Meter, was 55 ± 5 foot candles.

Diatoms were introduced at an inoculation level of about 400 diatoms/ml. Simultaneously, a 10-microcurie aliquot of P^{32} as phosphate ion [2] in sea water was added so that the counting rate of one ml. of the initial solution was about 3000–4000 counts per minute as measured under an end-window Geiger-Muller tube with a window thickness of 1.4 mg./cm.^{2} and a geometry of nine per cent.

Daily radiochemical assays were made on the cultures to determine the phosphate content of the total solution, the diatoms, and the liquid medium freed of diatoms by the following method: one ml. of the whole solution (after continuous stirring for five minutes) was evaporated to dryness under an infrared lamp on a 10

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[2] The radioactive P^{32} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

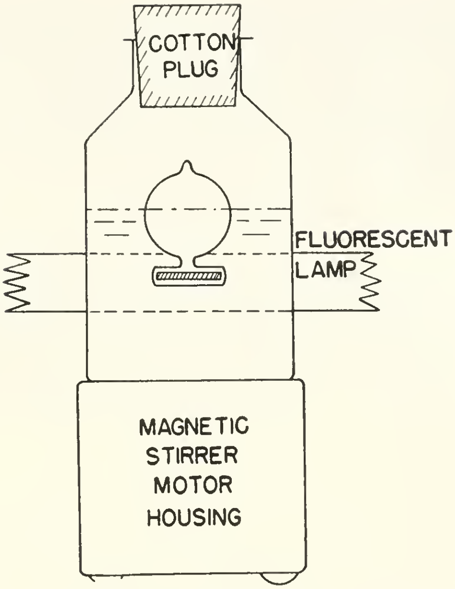

FIGURE 1. The diatom culture bottle. Six such bottles were aligned in front of a single fluorescent lamp for a single experimental run.

cm.[2] copper planchet. When the activity is plotted as a function of time, the known decay rate of 14.3 days of P^{32} is obtained (Fig. 2). This result indicated that there was no loss of phosphate during the experiment by adsorption on the walls of the glass vessel or by the clinging of diatoms to the walls or bottom of the culture flask. It further revealed that, within experimental error, the volume of diatoms at maximum growth was small with respect to the one-ml. aliquot, otherwise a dilution of the activity with time would have been observed.

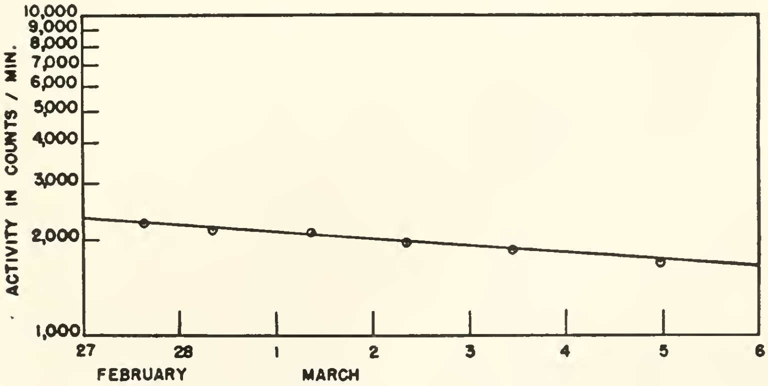

FIGURE 2. The radioactivity of the phosphate in an aliquot of the culture medium containing the diatoms is indicated by the open circles. The theoretical decay of P^{32} is shown by the heavy line. Data from experiment 3T.

Three ml. of the whole solution was centrifuged at 2000 rpm. and one ml. of the supernatant liquid was evaporated to dryness under an infrared lamp on a 10 cm.^{2} copper planchet. The activity of the supernatant liquid, a_s, was assayed under the Geiger-Muller tube and compared to the whole solution, a_t. We thus arrive at the value of phosphate per diatom, P_d, at any given time t, the time of withdrawal of the aliquots from the culture bottles as

$$P_d = \frac{P_t(1 - a_s/a_t)}{N}, \tag{1}$$

where P_t is the initial concentration of phosphate in the culture medium in microgram atoms/ml. and N is the total number of diatoms per ml.

The diatom population assay was made in the following way. One-ml. aliquots were withdrawn from the stirred cultures at the time that the samples for radiochemical analysis were taken. A sufficiently representative population of this

TABLE II

Culture flask	N, cells/ml.		P_s, micro-gram atoms/liter		Growth constant, hours	Final P_d, microgram-atoms $\times 10^8$
	Initial	Final	Initial	Final		
1L	318	122,000	12.8	4.4	15	6.9
2L	612	48,000		4.3	15	8.9
3L	1059	68,000		8.5	17	6.3
4L	450	76,000		5.4	13	9.7
5L	625	68,000		4.5	17	6.6
				Average with std. dev.		7.7 ± 1.5
10	1760	80,000	8.4	4.2	20	5.3
20	1970	40,000		4.4	20	10.1
30	2610	62,000		4.8	20	5.7
40	1320	54,000		4.0	16	8.2
50	1140	40,000		6.4	20	4.9
				Average with std. dev.		6.8 ± 2.2
1T	783	84,000	4.2	0.59	13	4.3
2T	327	72,000		0.57	11	5.0
3T	396	75,000		0.69	11	3.3
4T	420	43,000		0.76	12	7.7
5T	321	40,000		1.22	14	7.5
				Average with std. dev.		5.6 ± 2.0
1W	66	52,000	2.7	0.25	17	3.9
2W	253	30,000		0.25	12	5.0
3W	375	41,000		0.20	12	6.0
4W	429	52,000		0.15	12	4.9
5W	267	45,000		0.22	12	5.7
				Average with std. dev.		5.1 ± 0.8

was counted to obtain the density of the population expressed as cells per ml. Care was taken that the cells counted were normal and healthy. Moribund or dead cells were not numerous except in aged cultures, *i.e.,* in some cultures made in early experiments before the bio-assays were made.

The supernatant liquid of the radiochemical control flask was assayed radiochemically, and its activity was compared with that of the whole solution. Any difference would indicate phosphate precipitation. In all of the experiments the activity of the supernatant liquid and the whole solution of the radiochemical control flask were the same within experimental error.

The phosphate analyses were made by the Wooster and Rakestraw (1951) modification of the method of Deniges-Atkins on the solution before the addition of the radioactive phosphate solution. The maximum concentration of phosphate introduced by this addition was 0.01 micro-gram atoms/liter. The phosphate remaining in solution, P_s, at any time t is given by

$$P_s = (a_s/a_t)P_t. \tag{2}$$

Results

Diatom culture experiments were carried out in media varying in initial phosphate levels from 2.7 to 12.8 microgram-atoms per liter. Table II shows the results found between the diatom populations and the phosphate in solution. Columns 2 and 3 represent the initial and final cell count of the diatoms, the final count representing the population when the first signs of leveling-off appeared in the cultures. It should be noted in column 3 that essentially the same final concentrations of diatoms were obtained in all runs. Columns 4 and 5 represent the initial and leveling-off values of the phosphate remaining in solution. The growth constant, or the time for a given cell to divide, is given in column 6. The final concentration of phosphate per diatom as calculated from equation (1) is given in column 7. It is seen that the final phosphate content of the diatoms and the maximum cell population are essentially independent of either the initial inoculum or the initial phosphate level. The spread in the figures is undoubtedly due to cumulative errors in counting of the diatoms, radiochemical assay and sampling techniques.

If one takes as the phosphate content per diatom 5.0×10^{-8} microgram-atoms per cell and a population of 50,000 cells/ml., it is seen that 2.5 microgram-atoms of phosphate per liter are needed for a further division. In experiments T and W there was not enough phosphate remaining in solution to support much further growth.

The dynamic aspects of the phosphate uptake by diatoms are presented in Figures 3 and 4. Figure 3 depicts the relationship between the phosphate remaining in solution and the population level during an experimental run. It is seen that the point at which the diatoms reached their peak growth coincides closely with the leveling-off of the phosphate uptake at 0.25 microgram-atoms per liter. Evident in Table II, in the four replicate culture flasks of run W, is the leveling-off of the phosphate uptake and diatom growth which occurred at phosphate concentrations in the solution between 0.15 and 0.25 micro-gram atoms of phosphate-phosphorus per liter. It was further found that after the leveling-off period, the phos-

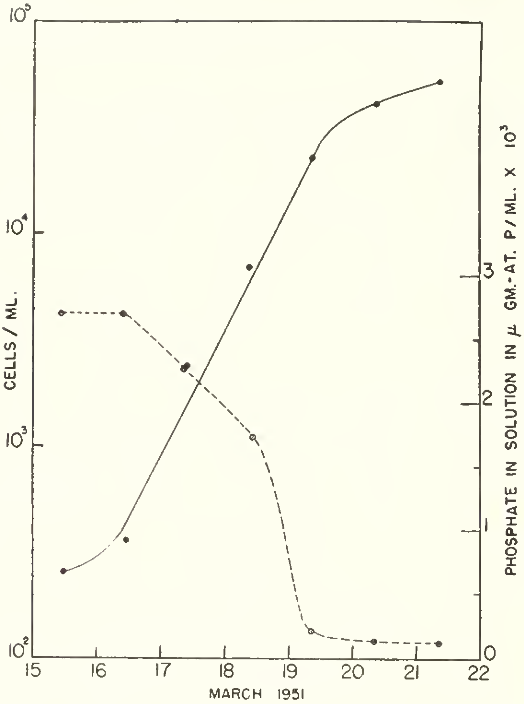

FIGURE 3. Phosphate depletion from solution in an actively growing culture. The heavy curve with solid points represents the exponential growth of diatoms, while the broken curve depicts the phosphate remaining in solution. Data from experiment 2W.

phate content was reduced even further. At this point the number of cells produced by a division is equal to the number that die or become dormant. But since death does not entail the immediate return of phosphate to solution, the available phosphate for uptake is only that which remains in a dissolved state. It was found experimentally that further divisions could take place even below 0.1 microgram atoms of phosphate-phosphorus per liter.

Figure 4 depicts typical relationships between the dissolved phosphate concentration and the phosphate fixed per diatom in a single experimental series. The phosphate content per diatom is observed to vary nearly linearly with the phosphate in the surrounding medium. We may thus write that

$$P_d = A + BP_s, \tag{3}$$

where A and B are constants. A would then be related to the minimal phosphate concentration per diatom, while B would be a function of the initial phosphate concentration for any given experiment. It should be noted that all points plotted in Figure 3 were taken while the diatoms were on the exponential growth curve.

To check the accuracy of the minimal phosphate content per diatom as determined radiochemically, diatoms were grown in the nutrient medium with no added phosphate. The diatoms were assayed daily for population density. At the

TABLE III

Culture flask	Final cell population, diatoms/ml.	P_d, micro-gram-atoms/diatom $\times 10^8$
1	25,000	4.3
2	22,000	6.6
3	28,000	5.1
4	28,000	3.1
5	25,000	4.9
6	25,000	5.7

approach of the leveling-off period, the diatoms were filtered onto a Whatman No. 42 7-centimeter filter paper disc. The total phosphate content of the diatoms contained in the known volume of the culture flask was determined by digesting the filter paper in 5 ml. of a 2:2:1 nitric: perchloric: sulfuric acid solution until fumes of sulfuric acid appeared. The residue was diluted to 100 ml. and an aliquot assayed for inorganic phosphate. Blank filter paper was simultaneously treated to determine the correction factor due to phosphate contained in the filter paper and the reagents. The corrected results of P_d obtained by this procedure are listed in Table III. It is manifest that they may be compared favorably with the minimal values obtained by the radiochemical method. The initial phosphate concentration was 1.1 microgram atoms of phosphate-phosphorus per liter, indicating that essentially all of the phosphate was utilized in diatom production.

One series of experiments was conducted on phosphate regeneration. The di-

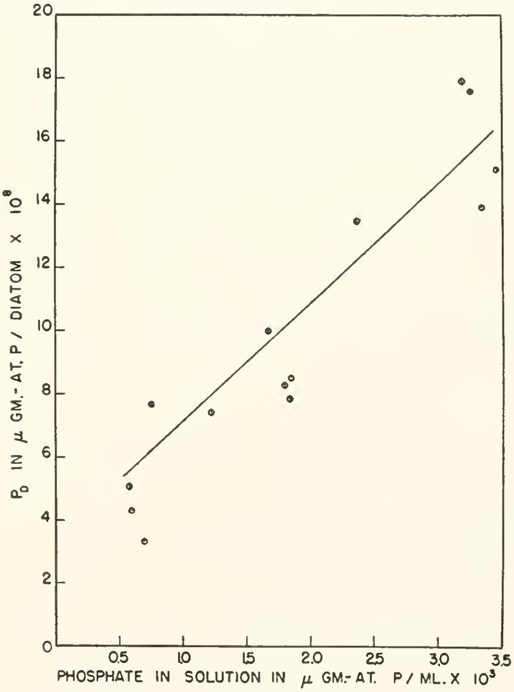

FIGURE 4. The phosphate fixed per diatom versus the phosphate concentration of the surrounding solution. Data from experimental run T.

atoms after reaching maximum growth were placed in a darkened box at 17° ± 2° C. with no stirring. Daily aliquots were taken and the supernatant liquid analyzed. As is shown in Figure 5, very little of the phosphate returned to solution after three weeks. This experiment was initiated at a low phosphate level. It would thus appear that the minimal phosphate is strongly bound. Although we may treat this experiment as somewhat of a laboratory phenomenon where the breakdown of the diatom is a function of the bacteria present, it nevertheless indicates the stable character of the bound phosphate. Further, in dynamic considerations considerably more time for death and decay of diatoms must be allowed for the return of phosphate to solution.

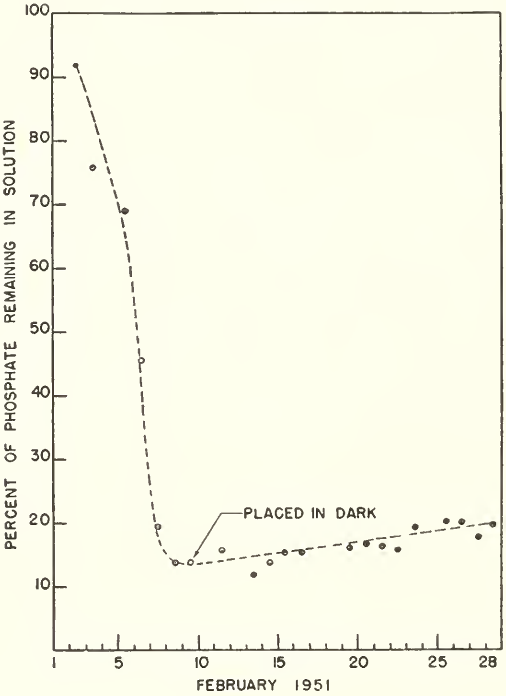

Figure 5. The regeneration of phosphate to the solution by diatoms deprived of light. The rapid uptake from February 2 to February 9 represents the diatom growth. Data from experiment 2Q.

In some initial experiments involving diatoms containing more than the minimal amount of phosphate, the phosphate per diatom was determined by collecting a known number of organisms on filter paper, and subsequently assaying radiochemically under the Geiger-Muller tube. However, in this process, it was found that washing the diatoms with radiophosphate-free sea water, to free them of any adhering phosphate in the nutrient medium, removed as much as 50 per cent of the phosphate from the diatoms. Thus a significant fraction of the phosphate above the minimal amount appears to be loosely bound and readily exchangeable, and we may designate it as the labile phosphate.

Discussion

From this dynamic study of the assimilation of phosphate by the marine diatom *Asterionella japonica,* it is evident that these plants have a phosphate content dependent upon that of the environment. This phenomenon, not heretofore fully established, adequately explains the anomalies in phosphate content of diatoms in both cultural and natural populations. Lund's experiments on the fresh water species *Asterionella formosa* first suggested this relationship which he designated as phosphate storage. He found that cells grown in stock culture of 74 microgram-atoms of phosphate-phosphorus per liter contained 3.7×10^{-8} microgram atoms of phosphate-phosphorus per diatom, whereas the cells in stock cultures of only 19 microgram-atoms of phosphate-phosphorus per liter contained but 1.5×10^{-8} microgram-atoms of phosphate-phosphorus each.

By these radiochemical techniques the minimum phosphate content of a diatom can be ascertained, which in the case of the species studied was about 5×10^{-8} microgram-atoms of phosphate-phosphorus per cell. This phosphate may best be designated as "bound phosphate" or the phosphate which is not readily exchangeable with that of the surroundings. Such a condition as this must obtain whenever the phosphate is incorporated irreversibly into the protoplasm. The labile or readily exchangeable phosphate should in no sense be considered as storage since this phosphate level reflects the environmental phosphate through the uptake process.

Inasmuch as this single species was able to flourish in phosphate concentrations lower than the minimum levels found in the euphotic zone of the Pacific Ocean and in surface sea water to which no phosphate had been added, it appears that the inorganic phosphate content of marine waters is representative only of the total potential population that it could support, assuming no diffusion of this nutrient. From the data in Table II of the culture experiments it can be seen that there is a limiting growth factor other than phosphate or nitrate. Moreover, sea water media enriched a second time with all nutrients or with all nutrients but phosphate and nitrate failed to produce growth of diatoms. Using this same technique on other essential elements, the authors hope to establish further minimum mineral requirements of this diatom.

The relationship between phosphate uptake and the concentration of nitrate can be qualitatively seen from the experimental data. The initial nitrate concentration in all experiments was constant at an excess of 2000 microgram-atoms per liter. From Figure 4 we see that initially the phosphate per diatom is about 16×10^{-8} microgram-atoms phosphate-phosphorus per cell, where the phosphate in solution is 3.5 microgram-atoms phosphate-phosphorus/liter. From Table II, experiment 3T, we find that at a final phosphate concentration of 4.2 microgram-atoms phosphate-phosphorus/liter, the cell content is about 5×10^{-8} microgram-atoms of phosphate-phosphorus. Thus, a high nitrate/phosphate ratio is reflected in a high phosphate content of the diatom cells. This result is in agreement with the work of Ketchum (1939).

In the present study it was not considered essential or possible to have bacteria-free diatoms. First of all, only the exponential growth phase of diatom populations was studied. Accordingly, the biomass consisted almost entirely of actively growing plants. There was thus no substrate for bacterial growth. The remarkably close correspondence between the curves of phosphate depletion and diatom growth would only result if the phosphate was entirely assimilated by the diatoms. The culture medium was autoclaved to remove any other competing living forms.

The diatom stock cultures which were used for inoculation were never freed of bacteria, inasmuch as they were healthy and normal cells. It seemed unlikely that any number of repeated washings could free these cells from bacteria which were attached between the frustule and the cell wall. Observations on decaying diatoms showed that the decay takes place within the frustule and the frustule does not open until the decay process is nearly complete. The standard procedure for determining sterility in diatom cultures is normally carried out on cells which have been repeatedly washed and never on aged cultures. It would seem that the only test for sterility should be one of direct observation as to whether the moribund cells decay.

SUMMARY

1. Studies were made on the uptake of phosphorus by the marine diatom *Asterionella japonica*, utilizing radioactive phosphorus as a tracer.

2. The higher phosphate concentrations in the solution were linearly related to higher contents of phosphate in the diatom cells.

3. A minimal cell concentration of phosphate for further growth was established for this species.

4. Two types of phosphate were found in the cells: the minimal content which was strongly bound and not removed by water washing of the diatoms and a labile phosphate which could be removed. Experiments on the release of phosphate conducted on diatoms containing only minimal amounts of it, indicated that only a small percentage of the phosphate was returned to solution as soluble phosphate after three weeks.

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THE EFFECT OF MALEIC HYDRAZIDE ON THE EMBRYONIC AND LARVAL GROWTH OF THREE AMPHIBIANS

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Since Schoene and Hoffman (1949) first reported that maleic hydrazide (MH) inhibits plant growth, many investigators have extended and in general substantiated their findings. Greulach and Atchison (1950) reported that MH inhibits mitosis in onion root tips, Compton (1951) that it inhibits mitosis in pea seedlings, Girolami (1951) that it inhibits mitosis in flax, and Greulach (1952) that it inhibits mitosis in bean buds. Darlington and McLeish (1951) found that MH inhibited mitosis in the roots of several species of plants, and that it induced chromosome breakage in *Vicia faba,* but not in *Rhoeo discolor, Muscari palmosus,* or *Scilla sibirica.* The breakage was confined to the heterochromatin (instead of to the euchromatin as with X-rays), and no sticky chromosome surface was produced.

Since MH has a striking effect on cell division and growth of plants it would be of interest and value to learn whether MH has similar effects on animals. There is very little published information on the effect of MH on animals. Zukel (1951) reports that the oral LD 50 of MH to rats is 2.2 gm./kg. body weight, and that there is no irritation to the skin or mucous membranes of experimental animals from contact with MH. An earlier mimeographed release from the Naugatuck Chemical Company (producers of MH) stated that acute toxicity of MH to mice by intraperitoneal injection was 1.5 gm./kg. of body weight. Johnson, working at the University of North Carolina, has unpublished data which indicate that MH does not affect the growth of mice nor the growth of testicular tumors in mice. Young white mice sprayed daily for three weeks with six different MH concentrations ranging from 0.001 to 0.2 per cent, or given drinking water with the same concentrations of MH for three weeks, grew as rapidly as the controls and exhibited no toxic symptoms. Older white mice into which testicular tumors were transplanted were injected subcutaneously for ten days with 0.1 ml. of one of nine MH concentrations ranging from 0.05 per cent to the undiluted original solution containing 30 per cent MH by weight. No concentration of MH inhibited growth of the tumors, nor did any concentration produce symptoms of toxicity.

EXPERIMENT WITH RANA PIPIENS

Ovulation was induced in female frogs (*Rana pipiens*) by pituitary injections, and the eggs were fertilized by sperm from testes macerated in Ringer's solution. Two days later, when the embryos were in the neural tube stage, 15 embryos were placed in boiled and cooled tap water, 15 in a 0.1 per cent solution of MH, and 15 in a 0.05

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[1] The authors wish to thank Dr. H. E. Lehman of the Department of Zoology, University of North Carolina, for suggestions concerning the experiments with Amblystoma.

per cent solution of MH. The MH was used in the form of the diethanolamine salt and was dissolved in boiled and cooled tap water, the same being true of the other experiments reported here. After 48 hours the embryos were transferred from the MH solutions to tap water and the embryological and larval development were observed over a period of six weeks.

Five days after the beginning of treatments the mean overall length was 10.5 mm. for the controls and both treated groups. The length of the body without the tail was 3.9 mm. for the controls, and 3.6 mm. for both treated groups. The body width was 3.2 mm. for the controls, 2.5 mm. for those treated with 0.05 per cent MH, and 2.4 mm. for those treated with 0.05 per cent MH, and 2.4 mm. for those treated with 0.1 per cent MH. These differences were not statistically significant. There was no observable effect of 0.05 per cent MH, but in the 0.1 per cent MH treatment there were retardation of eye development, smaller myotomes and gills, and a more sluggish response to light and touch than in the controls. Circulation as observed under a binocular dissecting microscope was apparently normal in both treated groups. The mortality rate in the 0.1 per cent MH was slightly higher than in the other two groups, but in the survivors the early minor differences in growth rates were overcome by the end of the experiment.

EXPERIMENT WITH BUFO SP.

Freshly laid eggs of an unidentified species of toad were collected in the field, and 75 embryos in the early blastula stages (with ectodermal epiboly incomplete) were selected and placed in 0.02 per cent MH, 75 similar embryos being placed in boiled tap water. After 48 hours the embryos in MH were transferred to tap water. Five days later four larvae from each group were selected for tail tip amputation and study of the rate and amount of tail tip regeneration, the remainder being retained for observations of growth and development.

The MH had no observable effect on embryonic or larval development, reflexes, or amount and rate of tail tip regeneration.

EXPERIMENTS WITH AMBLYSTOMA PUNCTATUM

Embryos of the salamander, *Amblystoma punctatum,* were obtained in early cleavage to yolk plug stages. The jelly was removed and batches of embryos were placed in tap water which had been standing 48 hours, and in 0.5, 1.0, and 2.0 per cent solutions of MH. After 10 days all the gastrulae were transferred to standing tap water and their development was observed over a period of several weeks. In a second experiment the posterior third of the tail was amputated from a group of larvae 5 days after hatching. The larvae were divided into three equal groups and placed in tap water, 0.25 per cent MH, or 1.0 per cent MH. After 10 days the regenerated tail tips were clipped and then fixed and stained by the Costello-Henley (1949) method. The regenerated tissue was examined under oil for possible effects of the treatment on mitosis, and the number of mitotic figures per tail tip was determined, there being 8 control tail tips and an equal number in each of the two treated groups.

In the first experiment all the embryos in the 2.0 per cent MH died within 50 hours, but 35 per cent of those in the 1.0 per cent MH and 70 per cent of those in the 0.5 per cent MH survived through the 10 day treatment period. Since only 84 per cent of the controls survived, the 0.5 per cent MH was apparently not particularly toxic. There was no superficial morphological difference between the controls and the surviving treated embryos, nor any significant difference in size. In the second experiment there was no significant difference in the size of the regenerated tail tips in the various groups. The mean number of mitotic figures per regenerated tail tip was 8.5 in the controls, 2.6 in the 0.25 per cent MH, and 3.0 in the 1.0 per cent MH. The differences between both treated groups and the controls were statistically significant at the 1 per cent level, but the two treated groups did not differ significantly from each other. There were many pyknotic nuclei in both treated groups, but none in the controls. All concentrations of MH used promptly killed the Daphnia and Cyclops introduced into the cultures for use as food by the salamander larvae.

Discussion

The results of these experiments indicate that MH does not have any significant or persistent effects on the growth and development of amphibian embryos and larvae similar to its effects on the growth of plants, even though several of the concentrations used on Amblystoma were much in excess of the concentration range of 0.02 per cent to 0.2 per cent usually used on plants.

In these experiments all concentrations of MH used were lethal to Daphnia and Cyclops, the 2.0 per cent MH was lethal to Amblystoma, and the 1.0 per cent MH was toxic to Amblystoma. The 0.1 per cent MH was somewhat toxic to Rana. Except for the latter concentration, all these concentrations were much in excess of those commonly used on plants. Taken in conjunction with the toxicity studies on rats and mice reviewed in the introduction to this paper, these data indicate that MH is toxic to animals only in rather high concentrations, considerably in excess of those used on plants. However, this does not take into consideration possible cumulative or delayed toxicity symptoms of lower concentrations. The sluggishness in some of the treated larvae of both Rana and Amblystoma could possibly be due to a decrease in the rate of respiration, since there is evidence that MH may decrease the rate of respiration in plants.

The data from the second experiment with Amblystoma would seem to indicate that MH did reduce the rate of mitosis in the regenerating tail tips. However, in the absence of any significant effects on total growth, in view of the fact that the two different MH concentrations had no different effect on the number of mitotic figures, and since starvation is known to induce pyknosis and inhibit mitosis in regenerating tail tips, the writers are of the opinion that the reduction of mitosis was not due to a direct effect of MH as in plants, but to starvation resulting from the lethal effect of MH on the crustaceans used by the Amblystoma larvae as food. Amblystoma larvae ingest only live, moving prey. It is planned to repeat this experiment with the addition of a starvation control group and with a wider range of MH concentrations, but in view of the basic complication involved it may be necessary to use other animals and techniques to clarify completely the effect of MH on mitosis in animals.

SUMMARY

1. A 2.0 per cent solution of maleic hydrazide was lethal to all *Amblystoma punctatum* larvae within 50 hours, but 35 per cent of the larvae survived 10 days in 1.0 per cent MH and 70 per cent in 0.5 per cent MH, while 84 per cent of the controls survived. All concentrations of MH from 0.25 per cent upward killed Daphnia and Cyclops introduced into the Amblystoma cultures as food. A 0.1 per cent solution of MH was slightly toxic to *Rana pipiens* larvae.

2. MH concentrations ranging from 0.02 per cent to 2.0 per cent had no significant effect on the growth of embryos and larvae of Rana, Bufo and Amblystoma. The full range of concentrations was not used on any one of the three species.

3. The number of mitotic figures in regenerating tail tips of Amblystoma was significantly less in those treated with either 0.25 per cent or 1.0 per cent MH than in the controls. Pyknosis was common in the treated tail tips but was absent in the controls. The writers are of the opinion that these are indirect, rather than direct effects of the MH treatment, and can be attributed to starvation following the death of the Cyclops and Daphnia provided as food for the Amblystoma larvae.

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THE METABOLIC RATES AND BODY TEMPERATURES OF BATS[1]

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Bats are among the hibernators—those mammals that pass the winter in a profound physiological state, characterized by: (1) reduction of the body temperature to virtually the ambient temperature, (2) reduction of the metabolic rate to an extremely low level, (3) reduction of other body processes (heart rate, respiratory rate, etc.) to minimal levels. These mammals are properly called heterotherms, for they are essentially poikilothermic when hibernating, but when awake and active maintain roughly constant body temperatures comparable to those of typical homoiotherms. It has long been known that bats reduce body temperature and metabolic rate whenever they become inactive (Hall, 1832). This reduction of metabolic rate would seem to be of great advantage to an animal that must expend such great amounts of energy as bats in flight must require. Pearson (1948) says: "Apparently because of small size and strenuous activities many bats have resorted to a metabolic parsimony of periodically lowered body temperature that allows their expensively gathered fuel to nourish them longer."

Despite the realization among naturalists that this remarkable condition exists in bats, there has never been any thorough study made to measure its dimensions, or to evaluate the possible impact on the lives of bats. The temperature coefficient of oxygen consumption has been studied in isolated tissues, in oxidative enzyme systems, in poikilothermic animals, and in newborn young mammals that have not yet developed the ability to regulate their body temperature. But it has not been possible previously to measure metabolic rate or oxygen consumption over a range of 40° C. in an intact, adult mammal. This paper presents the results of such measurements, made with the little brown bat (*Myotis l. lucifugus*) without subjecting the animals to thermal conditions outside of the range encountered in nature.

The investigation began by asking these questions: (1) To what extent do bats reduce their body temperature and metabolic rate during daily periods of inactivity? (2) Is there an essential physiological difference between this daily occurrence and hibernation? (3) Does this phenomenon separate bats from other hibernators on any physiological grounds? (4) What are the implications of this ability to reduce temperature and metabolism in relation to the life history of bats?

Question 4 will be answered in a later publication, as it is of greater interest in a mammalogical context than in a physiological one. However, the other three questions can be answered to a large degree here.

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I am indebted to many people for information and aid. Miss Mildred Wagner of this Center drew the figures. My wife, Ann, has read proof, checked references, and helped in innumerable other ways. Dr. P. F. Scholander gave counsel in the adaptation of his apparatus for this work. Dr. Laurence Irving, of the Article Health Research Center, has given encouragement and critical advice in the writing of this paper, and I am grateful to him for this and many discussions on similar matters.

To Dr. Donald R. Griffin, of Cornell University, I am most greatly indebted, for he first proposed that I do this research, and supervised, counseled, and criticized all phases of it. Furthermore, he has critically read both the thesis and this paper, and I have accepted nearly all of his suggested changes. However, all responsibility for statements made here is my own.

Materials and Methods

The bats used in this study were *Myotis lucifugus lucifugus* Kerr, the little brown bat. This species is found over much of North America, and is very common. Its habits are better known than those of any other American bat (Griffin, 1940; Wimsatt, 1945; Folk, 1940). It is found in hibernation in caves in northeastern United States during the winter months. It weighs from 5 to 9 or 10 grams.

Table I

Body temperatures of bats in relation to ambient temperature

Experimental temperature		Bat temperatures		Length of experiment	Rate of temperature change
Water bath	Air in vial	At start	At end		
° *C.*	° *C.*	° *C.*	° *C.*	*min.*	° *C./min.*
1.3	1.3	19.1	1.35	45	−0.39
23.0	23.1	24.7	23.2	30	−0.05
30.2	30.2	36.3	31.2	90	−0.05
37.0	35.5	35.7	37.5	15	+0.11

The bats used for these measurements were collected at caves in central Pennsylvania in winter, or in a large breeding colony near Ithaca, New York, in summer. Those bats taken in summer could be put into a torpid state even in July, merely by placing them in a refrigerator at 2–4° C. They were not fed during the time of their captivity, but were always kept in hibernation. High humidity was maintained in their quarters in order to minimize loss of water.

Apparatus. The determinations of oxygen consumption were made with a Scholander plastic semi-microrespirometer (Scholander, 1950). This had been modified for the present study by attaching the vials to the bottom so that the bats could hang in normal head downward position. Six bats could be measured at one time in the six separate units of this Lucite apparatus.

The body temperatures of some bats were measured with the bat in a similar-sized chamber. A small incision was made in the skin of the bat's abdomen, and a 30-gauge copper-constantan thermocouple inserted laterally along the body wall. This method of measurement of body temperature has been found to be quite close to the rectal temperature (Vernet and Metcalf, 1948). Reference junctions were in the air of the vial and in the water bath.

Procedure. The bats were removed from the cold box, weighed, and placed in the apparatus after an interval of time which was adjusted insofar as possible so that the bat would be placed into the glass vial with a temperature close to that at which the experiment was to be conducted. The experiments lasted for varying periods of time at the different temperatures, because when first placed in the chamber, the bat was active and consumed oxygen at a high rate. At 2° and 10° C., the steady state of oxygen consumption was not usually reached until eight hours had passed, while at 20° and higher it was reached much more quickly. At these higher temperatures death rates increased rapidly as the experiment lengthened, perhaps due to rapid depletion of already low nutritive reserves. Thus, the optimum length of the determinations at the various temperatures was selected at some point midway between the average time at which the steady state was reached and the average time of death.

The metabolic rate of interest in this study is obviously that of resting bats at various body temperatures. Hence the values obtained during the initial state of activity were excluded and the metabolic rates during the declining phase at the end of long experiments were also not considered to be relevant. All data presented below represent average values of oxygen consumption during steady state periods with no visible activity, periods within which the individual determinations of oxygen consumption show no extreme variation and no trend either upward or downward. In Table II is shown the average duration of this steady state period at various temperatures. As might be expected, much longer periods of constant metabolic rate could be obtained at lower temperatures.

TABLE II

Averages of oxygen consumption of Myotis l. lucifugus at various ambient temperatures

Ambient temperature	No. of bats	Average weight	Average length of steady state period	Minimum metabolism	Maximum metabolism	Average metabolism
° C.		gms.	hrs.	ml. O_2/gm./hr.	ml. O_2/gm./hr.	ml. O_2/gm./hr.
0.5	5	5.2	2	0.039	0.170	0.113
2.0	21	5.2	25.5	0.022	0.039	0.030
10.0	13	5.9	16	0.049	0.094	0.071
20.0	27	6.2	4.15	0.232	0.555	0.393
30.0	21	7.0	1.12	1.06	2.96	2.00
37.0	12	6.9	1.25	2.00	5.11	2.89
41.5	3	7.0	1.00	3.79	4.66	4.14
44.0	5	6.4	0.5	1.12	1.78	1.47

EXPERIMENTAL RESULTS

The data collected in this study have been plotted in Figures 1 and 2. In addition, all similar data from the literature have been collected, re-calculated to the present units if necessary and plotted in order to find whether the present data are near to the normal condition. The data on metabolic rate taken from other workers are not shown here.

Body temperatures. A primary point to be established in each measurement was the body temperature of the bat. It would have been difficult to conduct all

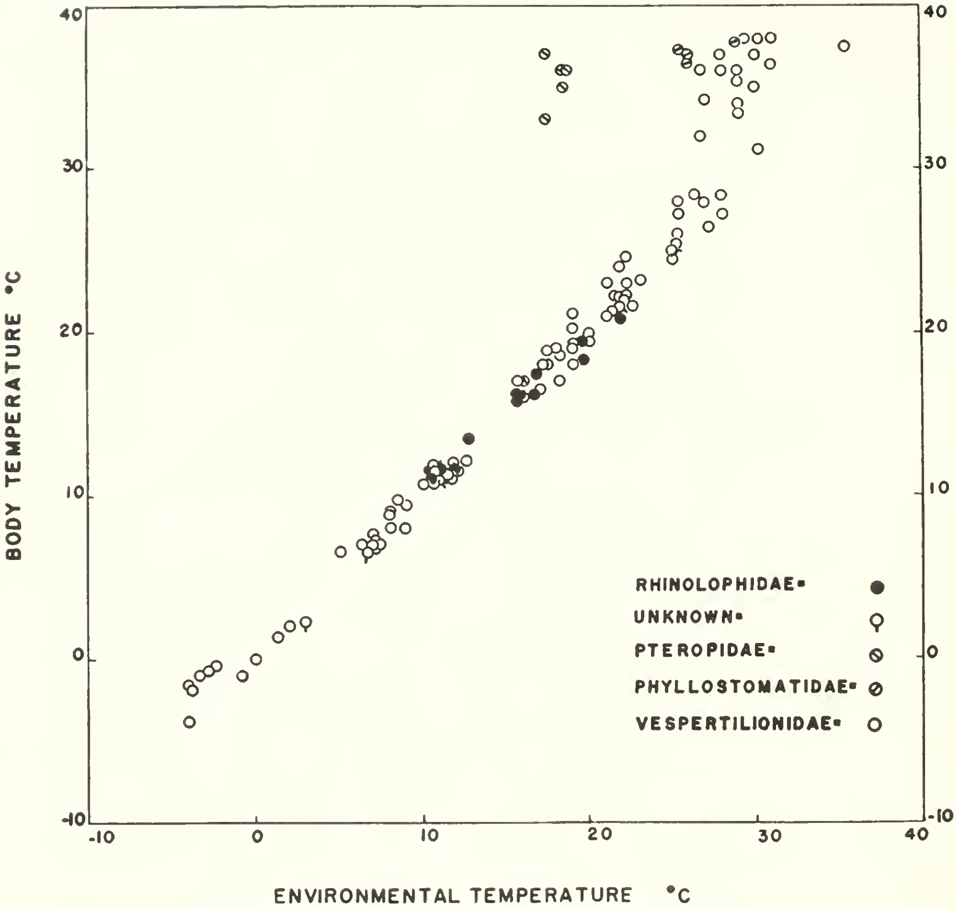

FIGURE 1. Body temperatures of bats in relation to environmental temperature. Data from the present study and all known literature. Four families, ten genera, and nineteen species are included. These are—Vespertilionidae—Hock, *Myotis lucifugus;* Reeder, 1949, *M. californicus;* Eisentraut (1933), *M. myotis, M. natteri, M. mystacinus, M. daubentonii, M. dasycneme, Nyctalus noctula, Eptesicus serotinus, Pipistrellus nathusii;* Eisentraut (1934), Delseaux (1887), and Kayser (1939), *Plecotus auritus;* Prunelle (1811), *Vespertilo murinus;* Burbank and Young (1934), *Nyctalus noctula;*—Rhinolophidae—Burbank and Young (1934), *Rhinolophus ferrum-equinum, R. hipposideros; —Pteropidae—*Burbank and Young (1934), *Pteropus geddei, P. giganteus, P. cotinus;*—Phyllostomatidae—Wislocki (1933), *Phyllostomus hastatus, Carrollia perspicillatum;*—Unknown—Pembrey and White (1896).

experiments with rectal thermocouples in the animals, particularly at the higher temperatures when the bats were alert and ready to actively struggle against any irritating attachment. Furthermore, a thermocouple attached to the body would undoubtedly have produced an increased metabolic rate in many of the animals. It was therefore decided to determine how closely the body temperature of bats approximated that of the water bath during the steady state period described above.

Accordingly, rectal temperatures were measured by copper-constantan thermocouples in bats at 4 water bath temperatures ranging from 1.3° to 37.0° C. The results are shown in Table I. It can readily be seen that by the end of the experimental period the rectal temperatures had come to a level closely approximating that of the water bath. The high initial rectal temperature at 1.3° C. bath temperature resulted from the fact that the bat was awake and warm when first placed into the apparatus; after 45 minutes, however, the rectal temperature had fallen to 1.35° C. and it seemed clear that the animal was in a condition comparable to the steady state period of the metabolic rate determinations.

In all cases there was a rapid approximation of the bat's temperature to the air temperature of the vial. This rapidity of change of body temperature increased as the difference between the original body temperature and the ambient temperature

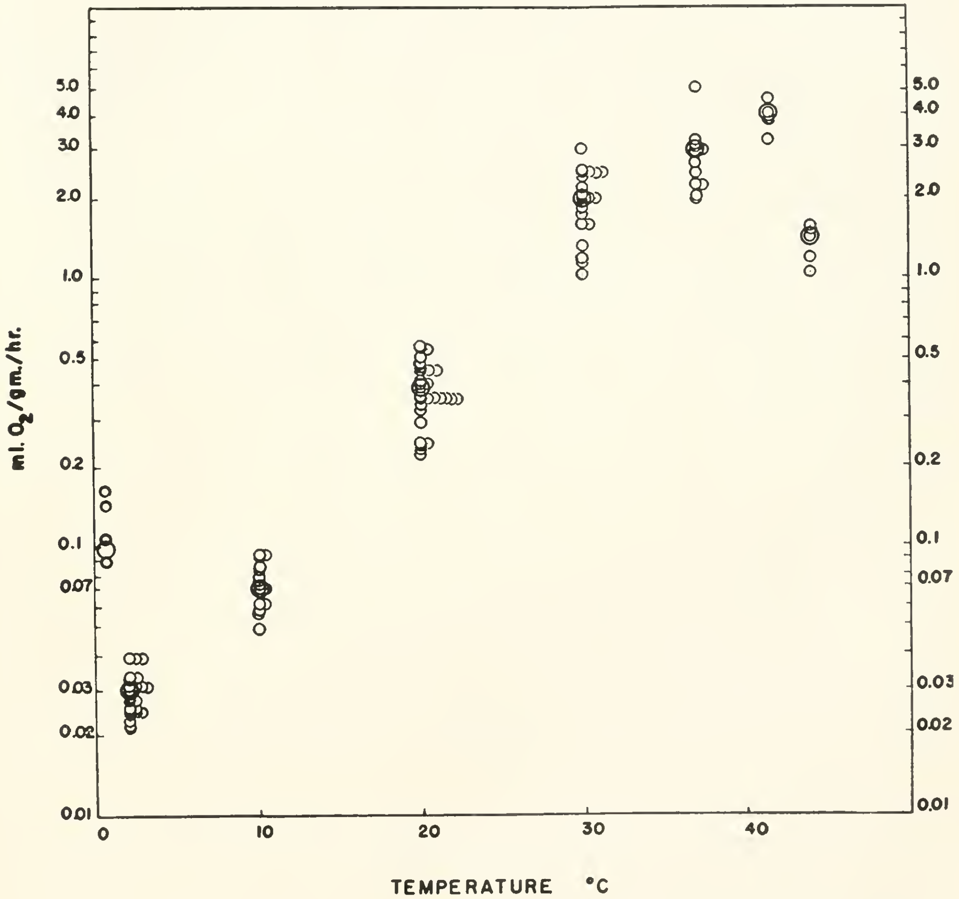

Figure 2. Semi-logarithmic plot of the metabolic rates of bats in relation to temperature. The temperature shown is environmental, but is very close to body temperature. Duplicate values are shown at the right of the line. Large circles are the average values.

increased. Table I shows these temperature determinations, including the rates of temperature change. It is apparent that body temperature closely approximates ambient temperature at all levels, but is closer at lower temperatures.

These results are in agreement with virtually all of the available information on body temperatures of bats. For example, Eisentraut (1934) found that the temperature of *resting* bats was nearly identical with the temperature of the surrounding air. Hence, during the steady state period of the present measurements, the body temperatures of the bats were, in all probability, less than 1 or 2° C. above the water bath temperature.

Figure 1 is a plot of all the body temperatures of bats that could be collected from this study and the literature, and that were not ruled out by the presence of activity at the time of the determination. It will be seen that there is an excellent approximation to a straight line. Furthermore, these data relate to several families and many genera of bats, and not only to a small closely related group.

Burbank and Young (1934) studied tropical fruit bats in Oxford zoos, and found that they maintained temperatures higher than that of the environment by means of shivering, flitting the wings, and other muscular activity. *Pteropus* sp. at room temperatures of 20° C. were always awake. At air temperatures of 17.5° to 18.5°, body temperature varied from 33.0° to 37.5° C.

Eisentraut (1938) studied temperature fluctuation in bats of the Cameroons. He found that the temperature at which they passed from a waking state to a lethargic state was *lower* than that found in temperate zone bats. However, he concluded that tropical bats behave in respect to daily reduction of body temperature just like the German bats he had studied. Further, he says, "Zusammenfassend lässt sich also sagen, dass die Beobachtungen an tropischen Fledermaüsen gezeigt haben, dass auch sie eine höchst unvollkommene Wärmeregulation besitzen, dass ihre Körpertemperatur und damit ihr Stoffwechsel weitgehend von Umgebungstemperaturen abhängig sind."

It thus appears that all bats exhibit a lack of thermoregulatory control when they become inactive, although they are capable of raising their temperature above that of the environment.

Metabolic rate. It may be expected from the above discussion on body temperature of bats that they will exhibit a variable metabolic rate dependent on the ambient temperatures to which they are subjected. Bats were exposed to ambient temperatures varying from 0.5° to 44.0° C., and their oxygen consumption measured. Figure 2 shows the results of these experiments. Each point represents the average oxygen consumption of one bat for the duration of the steady state period. Table II gives the average of all the steady state metabolic rates at each temperature, and gives also the average length of the period, the number of bats tested, and the average weight of the bats used.

The metabolic rate is at its minimum at 2° C., and is approximately double that value at 10° C. This is the range of temperature which Eisentraut (1934) characterizes as "deep hibernation." At 20° C., metabolic rate is five times that found at 10°, and by 30° there is another five-fold increase. Eisentraut (1934) delimited his "Tageschlaflethargie", or light hibernation as from 10° to 28° C. From 30° to 37° there is a smaller increase (1½ times) reflecting perhaps the fact that the bats are awake at the lower limit, although not yet with a "normal" body temperature. From 37° to 41.5° C., metabolic rate again increases 1½ times. This temperature was selected for study because it has been stated to be the body temperature during flight (Burbank and Young, 1934). Furthermore, Barkow (1846) found body temperatures of bats when awake to be 41.0° to 41.6° C., although he did not say whether they were flying or not. At 44° C., the metabolic rate was lower than at 41.5°, and death occurred quickly. It thus appears that bats live on a narrow limit of temperature tolerance at the upper level, although there is a very great tolerance at the lower limits. However, the fact that cooling by evaporation could not be accomplished in the vials of the apparatus to the degree possible in the wild must be considered as a possible factor in this rapid death.

At 0.5° C., the metabolic rate quadrupled from that found at 2°. The bats thus increased metabolic rate as the temperature approached the freezing point, even though they were in deep hibernation. Lyman (1948) found that Syrian hamsters responded to temperatures of −1.5° to 1° C. in three ways; the majority increased oxygen consumption and cheek-pouch temperature, and finally awoke; two hamsters increased oxygen consumption to a steady rate 3–4 times that recorded before ambient temperature was reduced, and maintained a cheek-pouch temperature 1.7° to 3.4° C. above that of the environment for as long as 22 hours; four animals died in hibernation, perhaps due to poor condition of the animals. Note that these are the three possible reactions of hibernators to temperatures below 0° C.: to awake, to increase metabolic rate and body temperature without awaking, or to die.

Johnson (1929) subjected ground squirrels to temperatures near freezing and found that in many cases they awoke, and then returned to hibernation at these low temperatures. Some squirrels did not awake, however, but died when the temperature was at or below 0° C. Wyss (1932) found that dormice showed a great increase in metabolism below 0° C., while the body temperature was at least 2° above freezing. Dubois (1896) and Benedict and Lee (1938) reported conflicting results with marmots. Dubois states that they *always* awoke when subjected to near zero temperatures, while Benedict and Lee say that near zero temperatures *never* awakened their marmots.

Unfortunately, the experiment with bats at 0.5° was not continued long enough to show whether or not the bats would have awakened. One of the five bats tested did not increase its metabolism above the range of values found at 2°, while all others went much beyond this. It may be that these four would have awakened in time, while the one that did not increase metabolic rate would have died. It is interesting to note that Eisentraut (1934) has recorded body temperatures of bats as low as −3° C. Kalabuchov (1933) subjected bats to temperatures of −5° to −17° C., and reported that if freezing did not reach the vital organs (lungs, heart, etc.) the bats could be revived by thawing. It would be desirable to know the metabolic rates of bats under such conditions.

DISCUSSION

Comparison with other data on bats. Metabolic rates of bats have been determined by several authors, either as oxygen consumption (Almeida *et al.*, 1926; Kayser, 1938, 1939, 1940; Morrison, 1948; Pearson, 1947), or carbon dioxide production (Delseaux, 1887; Koeninck, 1899; Pembrey and White, 1896), or both (Burbank and Young, 1934; Hari, 1909). These have been measured over a small range of temperatures with the exception of the series by Kayser (1940). The range covered in his study is from 4° to 30° C. This is a study of *Nyctalus (Vesperugo) noctula,* and although the number of determinations at any one temperature is small, the study is comparable to the present series of measurements. (Kayser's series on *Pipistrellus* covers a range from 5° to 17° C., and contains more figures at each temperature. There is, however, such great variation that it cannot have involved resting bats in all cases. At 5°, for example, oxygen consumption varies as much as 150:1). I have not used Kayser's mean figures, because it seems that he excluded some data that are admissible from the point of view of my study.

When the data for *Nyctalus noctula* (Kayser, 1940) are compared with those for *Myotis lucifugus* as ml. 0_2/gm./hr., very close correspondence is found at the lower temperatures (2° and 4°, 10° and 12.5° C.). At 20° there is a fair correspondence between the two series, but at 30° the rate for *M. lucifugus* is four times that found for *N. noctula.* Two explanations seem possible: (1) the sample used by Kayser was too small, and does not represent the average metabolism; or (2) there is indeed a difference in the metabolic rate in the two species. The latter possibility seems to be the more likely, for my *minimum* metabolic rate was higher than Kayser's maximum (not included in calculations). Further, it is well known that the metabolism of animals (of the same general type, *e.g.,* mammals) varies inversely with increased size, whether plotted as weight or as surface area. *N. noctula* is three to four times larger than *M. lucifugus,* and calculations from the mouse-to-elephant curve of Benedict (1938) indicate that the difference in the metabolic rates of these two species at 30° is not far from the expected divergence for their weight variation. It may, however, be significant that no difference could be detected in metabolic rate per gram at lower temperatures.

Comparison with data on other hibernators. There are many studies of the temperature and metabolism of heterotherms during hibernation and when awake. Dubois (1896) found no fluctuation of the body temperature of marmots and other hibernators except in the fall, immediately preceding hibernation. Johnson (1928), on the other hand, states that ground squirrels (*Citellus* sp.) showed temperature fluctuation at all times of the year. When not hibernating the range of temperature was 32° to 41° C. The lower temperatures graded into those of slight torpor at about 29° to 32° C. Johnson (1931) considered that these fluctuations of body temperature in response to changes of ambient temperature were due (in part, at least) to the animal's "rather poorly adjusted heat regulating mechanism." Hamilton (1934) observed that the body temperatures of nine woodchucks varied from 34.9° to 40.0°, with the average 37.4° C. It thus appears that there is a lability of the body temperature of these heterotherms which extends over a small range, even when the mammal is not hibernating.

Kayser (1939) studied the effect of variations in ambient temperature upon the respiratory exchange of hibernators when they were awake. He used hedgehog, hamsters, marmot, spermophile, and dormice, as well as bats. All of these animals, with the exception of the bats, were capable of thermoregulation, although not to the same degree of precision as that found in the homoiotherms. Kayser also found that the basal metabolism of hibernators when awake was not distinctly different from that of the homoiothermic mammals.

Conclusions. It is now proper to sum up the evidence offered, in an attempt to answer the questions posed in the introduction. First, it is evident that bats can greatly reduce metabolic rate and body temperature. However, the normal daily reduction has not been indicated thus far. The answer seems to be that these physiological measures of energy production are reduced to a level dictated by the ambient temperature. For a bat resting in 20° C., therefore, body temperature will be close to 20°, and metabolic rate will be near that indicated in Figure 2 for that temperature. This will be true at *any* ambient temperature, as long as the bat is in a resting state.

Question 2 asked whether there was a physiological difference between hibernation and the daily reduction of body temperature and metabolic rate. Hall (1832) called this latter state "diurnation," in contrast to "hibernation." Eisentraut (1934) called it "Tageschlaflethargie," or "leichter Lethargie," in contrast to "Winterschlaf" or "tief Lethargie," and considered that 10° C. was the dividing line between these two states. It does not seem to me that there is any essential distinction between them, and that those differences that do exist are those of degree, not of kind. To be sure, metabolic rate is greatly increased during exposure to temperatures above 10°, but this alone does not warrant erection of a new state, or the dignity of a new term. Rather it appears that the increased metabolism at these intermediate temperatures is a reflection of the increased function of tissues and organs of the bat, although it is still not capable of locomotion. In this respect, bats may be compared to the frogs, toads, and curarized dog of Krogh (1914), all of which showed an increase in metabolism at 20° although no movement occurred. To summarize, no basic physiological distinction can be shown to exist between the conditions found during the daily sleep of bats and those found during hibernation.

It seems apparent that the heterothermic mammals (other than bats) when not hibernating have physiological characteristics much like those of their non-hibernating relatives. Body temperature varies over a greater range, and metabolism reflects this, but both are in nearly all respects closely similar to the phenomena manifested by homoiotherms. Bats differ from other heterotherms in that their body temperature when at rest *at any season* always approximates that of their environment. The metabolic rate reflects this change, so that the metabolism is in direct proportion to the body temperature at all times. This condition is in opposition to that found in homoiotherms, where metabolism varies with external temperature, while body temperature remains nearly constant. Even other hibernators do not exhibit this phenomenon, for their metabolic rate does not vary in relation to body temperature when they are not hibernating. Rather, the metabolic rates during hibernation are of a quite different magnitude than those found during the normal state, and only these levels are clearly demonstrable, for when passing into or out of hibernation the metabolic rate at any one body or ambient temperature is a transitory affair (Lyman, 1948). Bats are thus the only mammals in which metabolic rate varies directly with body temperature. Question 3 must then be answered to the effect that bats are much different from other heterotherms in their lack of thermoregulation at all times of the year.

SUMMARY

1. Oxygen consumption was measured in resting little brown bats, *Myotis l. lucifugus*, at ambient temperatures from 0.5° to 44.0° C. Body temperature was shown to approximate the ambient level under the conditions of the experiments, so

that in effect the metabolic rate was measured over very nearly this range of body temperatures.

2. The function relating oxygen consumption to body temperature is not linear; the Q_{10} is 2.94 from 2° to 10° C., 5.54 from 10° to 20° C., 5.09 from 20° to 30° C., 1.69 from 30° to 37° C., and 2.22 from 37° to 41.5° C. The ratio between highest and lowest resting metabolic rates is 138:1; the temperatures represented are 41.5° and 2° C.

3. Some notes on the reaction to temperatures very close to 0° C. are included. Bats increased metabolism over that observed at 2° C. when exposed to near-freezing temperatures. The experiment was not continued long enough to find whether or not they would eventually awake.

4. The data are compared with the findings of other investigators using several species of bats. There is a close correspondence between body temperature and ambient temperature in all species, except that the tropical forms increase heat production by muscular work. Bats of the temperate zone appear to show the usual effect of size on metabolic rate per unit weight at temperatures of 30° C. or above, as Benedict (1938) has demonstrated occurs in a wide variety of homoiotherms. At 2° and at 10° C. this difference is not apparent in a comparison between these measurements and those made by Kayser (1940) on a much larger bat.

5. It is concluded that there is no physiological basis for distinguishing the daily reduction of temperature and metabolic rate in bats from that found in hibernation, except in degree. It appears that this daily phenomenon is due to a lack of thermoregulatory control.

6. Bats are considered as distinct from other heterotherms in that, at all seasons of the year, their resting temperature and metabolic rate is dependent on the ambient temperature. This also is due to their lack of thermoregulation. They are the only mammals in which the resting metabolism is a direct function of the body temperature, rather than of the ambient temperature. Nevertheless, when active, they can maintain body temperatures of 40° C. or above.

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STUDIES ON CELL–DIFFERENTIATION: THE ROLE OF AUXIN IN ALGAE, WITH PARTICULAR REFERENCE TO RHIZOID–FORMATION IN BRYOPSIS[1]

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Auxin, the plant growth-hormone, has been shown to occur in species of brown algae, red algae, green algae and diatoms (van der Weij, 1933; duBuy and Olson, 1937; van Overbeek, 1940a, 1940b; Thimann *et al.*, 1942). Judging by these reports auxin is ubiquitous in the algae, yet relatively few experiments in the literature deal with the possible role of auxin in these plants; the overwhelming majority of auxin-experiments have used seed-plant material. Most of the auxin-experiments on algae have been concerned with cell multiplication in unicellular Chlorophyceae. Leonian and Lilly (1937) found inhibition with 1 p.p.m. of indole-acetic acid (IAA) and toxic effects with 10 p.p.m. on the growth of five different species in medium containing sugar. Yin (1937) reported significant increases in average cell *diameter* of *Chlorella vulgaris* in the presence of 1 to 40 p.p.m. IAA and sugar, although he found "no conclusive differences (in) the total growth of the cultures as determined by . . . dry weight determinations." A "positive stimulating effect" of IAA and other growth-substances was reported in a brief note by Brannon (1937) for *C. vulgaris, C. pyrenoidosa* and an *Oocystis* sp. grown in sugar-free medium; he claimed to have "some evidence that growth substances accelerated the rate of cell reproduction and increased the size of cells." Pratt (1938), testing a wide range of IAA concentrations, found no effect on the average cell diameter of *C. vulgaris*, although the cell number per mm.^{3} was increased up to 32 times in sugar-free medium. Brannon's full report (Brannon and Bartsch, 1939) confirmed Pratt's finding of an increase in cell-number in *C. vulgaris* when IAA was added, but now reported that it was "impossible to secure reliable data respecting the effect of a growth substance upon the size of (*Chlorella*) cells." They pointed out that the inhibition from IAA reported by Leonian and Lilly (1937) was probably due to the use of dextrose in the culture medium, since Brannon and Bartsch found fewer cells per mm.^{3} with dextrose plus IAA than they did with dextrose alone. Lilly and Leonian (1941) complicated matters once more by reporting no increase in total dry weight of *Chlorella* cultures grown in the presence of 10 p.p.m. IAA but in the absence of dextrose. They state that there was a "fairly dependable parallelism" between dry weight and cell number. However, Brannon and Sell (1945) reported *increases* in dry weight of more than 4 times from the addition of the same concentration of IAA. Their culture of *Chlorella pyrenoidosa* was reportedly a duplicate of the culture called *C. vulgaris* by Lilly and Leonian (1941). Brannon and Sell obtained "pronounced and persistent variations" in preliminary experiments with IAA, variations which disappeared when the IAA was recrystallized to a constant melting point of 165° C.; they suggest lack of purification of IAA as a possible basis for the discrepant results of Lilly and Leonian. Inhibitory or toxic effects of IAA on cell number were reported by Manos (1945) for *C. vulgaris.* Unlike most of the other workers, Manos makes no mention of either adjusting the pH or of buffering.

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[1] This work was carried out during the summer of 1950 while a Lalor Fellow at Woods Hole.

An extensive paper by Algeus (1946) confirmed much of the earlier work and cleared up most of the discrepancies. The great importance of pH in IAA experiments was first pointed out: *Scenedesmus obliquus* and a strain of *C. vulgaris* showed an increase in cell number upon the addition of IAA only at acid pH, the effect of IAA increasing with decreasing pH. Furthermore, there were consistent differences in response to IAA not only between species but also between different strains of the same species. Only three of the ten species tested showed increases in cell number from any IAA concentration within the 0.1 to 100 p.p.m. range. For each of these three species the concentration giving maximum response was different. The observation of Brannon and Bartsch (1939) that IAA increased cell number in sugar-free medium but not in medium containing sugar was confirmed, as was Yin's (1937) report that IAA increased cell *size.* However, Algeus found cell size increased in sugar-free medium only at concentrations of IAA (100 p.p.m.) which were so high that the normal increase in cell number was inhibited. And only one of the two tested species showed even this increase.

In summary, a few species of unicellular green algae have been found to show an increase in cell number at IAA concentrations of the same order of magnitude as are effective physiologically in seed-plants. A larger number of unicellular green algae showed no increase in cell number even at acid pH, without added sugar, and with a wide range of applied IAA concentrations.

Auxin experiments with algae which are morphologically more complex have been even fewer. Olson and duBuy (1937) cited experiments to show that the polarity of the germinating zygote of *Fucus* could be determined by a high local concentration of IAA. They found no increase in the number of rhizoids formed when IAA was added. Young *Bryopsis* plants were treated with IAA in sea water at various molar concentrations by Darsie (1939), but the only effect observed was inhibition of growth at the 10^{-3} M and $10^{-4}M$ concentrations. It should be mentioned, however, that throughout his work Darsie deliberately restricted his attention to growth and tropisms of the shoot only. Kylin (1942) found that IAA increased the number of cells formed by germinating zygotes of *Ulva* and zoospores of *Enteromorpha* at pH 8.2, a result that has been confirmed with respect to *Ulva* by Levring (1945). Davidson (1950) applied a range of concentrations of various growth substances to fronds of *Fucus* and *Ascophyllum* and to *Fucus* sporelings. He reported stimulation of terminal frond growth, except with indole-butyric acid on *Ascophyllum* fronds, and stimulation of general growth of *Fucus* sporelings with IAA, indolebutyric and naphthalene-acetic acids. However, auxins in lanolin usually inhibited frond growth and never stimulated it. Auxins had no stimulatory effect on intercalary growth of fronds of either genus, no matter what the concentration or method of application. The fact that the "inhibitor" iodoacetic acid stimulated *Fucus* sporelings in essentially the same pattern relative to concentration as did IAA suggests the advisability of controlling pH effects. This was not done.

Williams (1949) reported that IAA produced gains in wet weight of small discs cut from some portions of the blade in *Laminaria.* Here, too, the published results are not unequivocal. There were no replications, no measures of variability; almost half of the IAA-treated samples disintegrated during the 16-day experimental period, and the largest wet-weight increase reported was only 18 per cent over the controls.

It appears, then, that while auxin is widely present in the algae, there is little evidence as to its role—if any—in algal morphogenesis.

It is reasonable to expect auxin to have somewhat similar roles in the various plant groups in which it occurs. Now, one of the most striking effects of auxin in flowering plants is the stimulation of root formation by high concentrations of auxin. The structure in algae which most closely corresponds to the root of a flowering plant is the rhizoid. Although the rhizoid is a mere protuberance from a single cell (instead of a multicellular organ with a very high degree of cellular differentiation and diversity like the root) yet the rhizoid is usually negatively phototropic, forms a close anchoring connection with the substratum, and is formed typically at the basal end of stem-like structures. These are characteristics which rhizoids share with roots. Hence, the following experiments were designed to determine if rhizoid-formation in algae is stimulated by auxin.

MATERIALS AND METHODS

Bryopsis plumosa (Hudson) C. Agardh was used as experimental material. *Bryopsis* was chosen because its normal life cycle is well known, some aspects of its developmental physiology and morphology have been studied (Noll, 1888; Winkler, 1900; Steinecke, 1925; Darsie, 1939), it has already been shown to contain relatively large amounts of ether-extractable auxin (Darsie, 1939; van Overbeek,

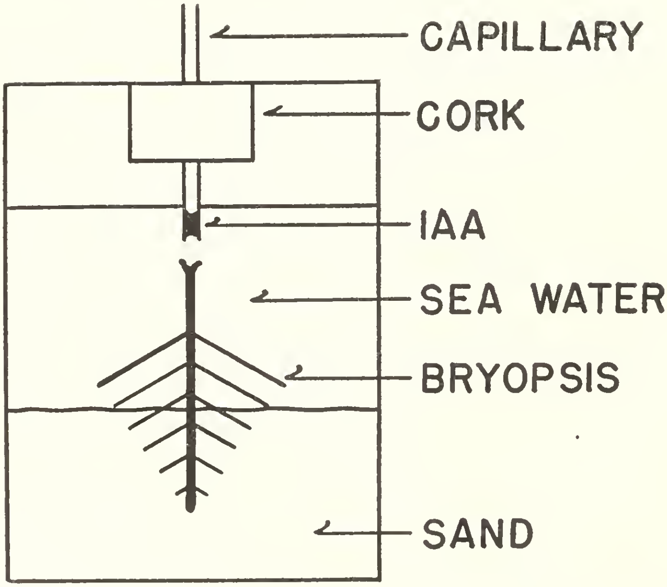

FIGURE 1. Diagram to show method of establishing a gradient of IAA along the long axis of *Bryopsis* plants.

1940a) and in its unicellular, multinucleate structure it seemed satisfactorily far removed from the organization of a flowering plant (Fritsch, 1935).

The general morphology of a young plant is shown diagrammatically in Figure 1. The vertically upright stem is positively phototropic, forms branches at the apex and negatively phototropic rhizoids at its base (Winkler, 1900; Steinecke, 1925). The chloroplasts are typically concentrated in the illuminated portions of the plant. The lumen of a young branch is in continuity with that of the main axis but by the time a branch is the 18th to 20th branch from the apex a cross-wall is beginning to form at its base. Thus, the branches furthest from the stem apex are morphologically separate cells.

Freshly collected, vigorous plants of *B. plumosa* were examined under the dissecting microscope and paired on the basis of close similarity in size, number of branches and rhizoids. One plant of each pair was placed in an inverted position in a shell-vial containing 10 ml. of sea water, as shown in Figure 1. The distal half of the branch-bearing portion was embedded in sand. A glass capillary was held so that the lower end was a few mm. above the base of the plant. The lower end of the capillary was filled with 0.7 to 1.0 mg. of IAA crystals. In replications of the experiment which used longer-stemmed *Bryopsis* plants, the lower opening of the capillary was held about 1 cm. above the oldest branches (*i.e.*, half-way down the unbranching portion of the main stem). The shell-vials shown in Figure 1 were placed in a water bath (at 21.5 ± 1° C.) to cut down convection currents which would disturb the diffusion gradient of IAA from the capillary tube. The relatively dim light from the windows was supplemented during the day with a 60 watt incandescent lamp held 18–24 inches above the shell-vials. The experiments lasted 12–15 days. Progressive dissolving of the IAA crystals occurred during the experimental period. There were usually some crystals left in the capillary even after 12 days.

To determine the extent to which the acid IAA changed the pH of the sea water, acetic acid was added to sea water to give the same molar concentration as was obtained with the highest concentration of IAA used (1.0 mg./10 ml. of sea water). (Acetic acid and IAA have the same pK.) The pH determinations were made with a Cambridge pH meter using a glass electrode. The untreated sea water had a pH of 8.01; addition of the acetic acid gave a pH of 7.25. Since all the IAA in the capillary was rarely dissolved by the end of each experiment and since the total possible drop in pH was so relatively small, pH effects were hereafter neglected.

At the end of the experiment the plants were first examined *in situ* with a horizontal microscope, then removed and compared under higher powers of the dissecting microscope.

Results and Discussion

When small, independently growing plants were prepared as described above, the results of all six replications showed that the superposition of an auxin gradient induces rhizoid-formation at the base of 77–95 per cent of the exposed (*i.e.*, aboveground) branches, while no rhizoids are formed at the base of branches in the control plants (Table I). Those branches which were below the level of the sand showed no basal rhizoids in either group of plants.

As *Bryopsis* plants continue their growth, secondary branches grow out on the primary branches and rhizoids usually develop at the base of the primary branches near their region of connection with the main stem. Eighteen of these primary branches with their array of secondary branches were dissected off the original main stem, paired and then treated as were the individual plants above. Results are shown in Table II. The data indicate that the auxin-gradient still has a stimulatory effect on rhizoid-formation although the secondary branches are not nearly as reactive as are the young primary branches.

These experiments verify the hypothesis that the external application of auxin acts in *Bryopsis* to induce rhizoids at the region of relatively high local concentration. The more extended hypothesis that auxin is the agent, naturally occurring in *Bryopsis*, which controls rhizoid-formation in the normal untreated plant is also supported by our experiments, particularly when they are considered in the light of reports of ether-extractable auxin in *Bryopsis* (Darsie, 1939; van Overbeek, 1940a) and of Darsie's finding that 73 per cent of the total plant auxin is in the basal (*i.e.*, rhizoid-forming) half of single-celled *Bryopsis* plants.

TABLE I

Effect of an external auxin-gradient on rhizoid formation in young Bryopsis plants.
(The results of two of the six replications are shown.)

IAA-treated		Controls	
No. of exposed branches	No. of branches forming rhizoids	No. of exposed branches	No. of branches forming rhizoids
13	10	24	0
19	18	20	0

A possible obscuring factor was that despite the surface cleaning done under the dissecting microscope, the *Bryopsis* plants were, of course, not sterile; hence, the possibility must be recognized that the treatment with auxin may have acted indirectly on *Bryopsis* through the other organisms present. In my opinion this is unlikely.

The following interpretation of the development of *Bryopsis* can be advanced, based on these studies and those of previous researchers. *Bryopsis* is an organism in which cytoplasm and chloroplasts can easily migrate from one portion of the coenocyte to another (Winkler, 1900; Steinecke, 1925). In the normal young plant auxin collects at the basal end of the coenocyte (Darsie, 1939) and there induces the formation of rhizoids. Once the older primary branches become morphologically separated from the main stem by the formation of thick cross-walls at their proximal end, the auxin produced in these branches can much less readily, if at all, move into the main stem. When the concentration of auxin becomes high enough at the proximal end of these separate branches rhizoids are formed there. Proximal rhizoids were never found in freshly collected material except when the "isolating" cross-wall was already present.

TABLE II

Effect of an external auxin-gradient on rhizoid formation by secondary branches of isolated primary branches

No. of pairs with more rhizoids on auxin-treated branches	No. of pairs with the same no. of rhizoids on each	No. of pairs with more rhizoids on the control branches
6	3	0

When *Bryopsis* is shaded (Winkler, 1900) the branch-tips continue their elongation as rhizoids instead of as "shoot" structures. Winkler (1900) and Steinecke (1925) have reported experiments which they believe show that this transformation is preceded by a migration of chloroplasts and cytoplasm out of the branch-tip and their replacement by cytoplasm which was in the rhizoidal end of the coenocyte. The experiments with auxin suggests that hormonal redistribution accompanies or is a consequence of the redistribution of cytoplasm.

Hence, it is suggested that one of the key differences between *Bryopsis* and seed-plants, the difference which is reflected in the strict developmental polarity of seed-plants and the labile polarity of *Bryopsis*, is that seed-plants have a strongly polar system for auxin-transport (Went and Thimann, 1937; Jacobs, 1950) while *Bryopsis* does not.

SUMMARY

1. Application of indole-acetic acid, a naturally occurring auxin, to young *Bryopsis* plants results in the differentiation of rhizoids at the proximal end of most of the exposed "branches." Control plants formed no basal rhizoids.

2. Similar results were obtained with respect to rhizoid formation on secondary "branches" when the primary branches were excised from the original main stem and exposed to auxin. The response was not as strong under these circumstances.

3. Considered with the fact that auxin is present in large amounts in *Bryopsis*, with most of the auxin being in the lower (*i.e.*, rhizoid-forming) half of the plant, these experiments verify the hypothesis that the formation and distribution of auxin control rhizoid-formation in *Bryopsis* in a closely analogous way to auxin's effect on root-initiation in seed-plants.

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OBSERVATIONS ON THE MORPHOLOGY AND LIFE-HISTORY OF MICROPHALLUS LIMULI N. SP. (TREMATODA: MICROPHALLIDAE)

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During the summer of 1950, Dr. T. H. Waterman, while studying the finer structure of the eyes of the horseshoe crab, *Limulus polyphemus,* observed small cysts, each of which contained a larval worm. Cysts containing living larvae were referred to me for study and possible identification. The wall of the cyst is very tough and it was impossible by mechanical means to liberate the larvae without injury. Some of the cysts were left in sea water and two days later two of them had ruptured, freeing the larvae which were alive and active. The worms were almost sexually mature; the testes and seminal vesicles contained motile spermatozoa. The excretory system was traced completely and the morphology of the metacercariae showed them to be a species of *Microphallus.* An abstract of the findings was presented at the summer meeting of the Marine Biological Laboratory (Stunkard, 1950).

Twenty-eight specimens of *L. polyphemus,* collected in Pleasant Bay, Orleans, Mass., and with a size-range from 5 cm. carapace length to fully grown individuals, were examined and all were infected. Cysts were present in the connective tissue of the digestive gland and other organs as well as in the eyes, and in all of them the larvae were apparently representatives of a single species.

Cysts were fed to baby chicks, half-grown white mice and young golden hamsters. One chick was examined 2 days and another 5 days after feeding, but no worms were found. Examination of the birds' feces did not yield eggs of the parasite and birds killed later than 5 days after feeding were free of infection. One mouse died 12–16 hours after the first feeding and many excysted metacercariae were found in the initial portion of the small intestine. Another mouse, killed 2 days after feeding, contained worms with eggs in their uteri and all of 6 mice examined 2–4 days after feeding contained mature worms. After 5 days the number of worms diminished and none were found after 9 days. No worms were found after subsequent feedings of metacercariae, indicating a possible immunity from the first infection. The feeding of cysts to golden hamsters gave results substantially like those with mice. From these feeding experiments and the rapid elimination of the worms, it appears that mice and hamsters are not natural or normal hosts of the parasite. However, the recovery of sexually mature specimens makes it possible to describe the species and compare it with other related ones. The specimens can not be assigned with certainty to any known species and accordingly they are described as a new species for which the name *Microphallus limuli* is proposed. Specimens must be designated in some way and since it is simpler and easier to drop a name in syonymy than to distinguish between different species which have been erroneously included under a single name, no serious inconvenience will result if the present specimens should eventually prove to belong to a previously described species.

Description

Cysts (Fig. 1) removed from *L. polyphemus* were oval, colorless, 0.16–0.2 mm. in length and 0.12–0.18 mm. in width. The wall was 0.009–0.011 mm. in thickness, hyaline, and resolvable into two layers, approximately equal in thickness. This wall was probably produced by material from cystogenous cells of the cercaria. It was enclosed in a thin, colorless, fibrous coat, probably deposited as a reaction product by the host.

Released from the cyst a metacercaria, under moderate coverglass pressure, measured 0.24–0.40 mm. in length and 0.10–0.20 mm. in width. Fixed, stained and mounted, this larva measured 0.30 by 0.19 mm. In it the testes, ovary and vitellaria had attained almost complete development; the testes and seminal vesicle contained motile spermatozoa. The size and location of the organs are shown in Figure 2. The flame cell formula is 2[(2 + 2) + (2 + 2)], which so far as known is characteristic of all members of the family Microphallidae. The cuticula bears imbricate, flattened spines which become progressively smaller posteriorly and may be absent behind the acetabulum. In living specimens the edges of the body tend to bend ventrally, forming a rim around an elongate depression. Typically there is a slight constriction at the level of the bifurcation of the digestive tract, and the body may become pyriform with either the anterior or posterior portion wider than the other. In front of the digestive ceca the body is filled with minute, unicellular glands.

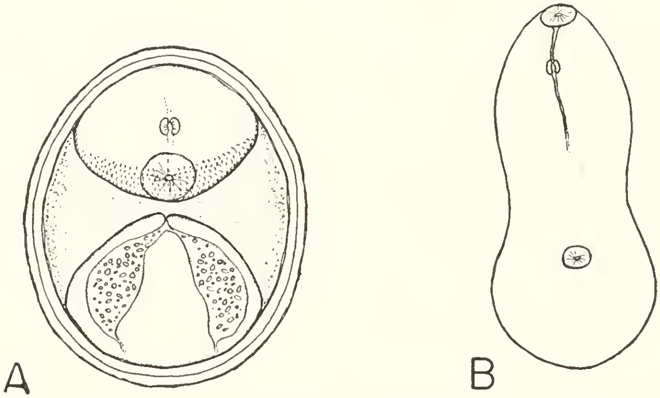

FIGURE 1. A; metacercaria in cyst, from *L. polyphemus*. B; outline drawing of fixed and stained specimen, 0.314 mm. long, with 84 eggs in the uterus, to show characteristic shape when elongate.

Sexually mature worms (Figs. 1B; 4) from mice and hamsters are very little, if any, larger than the metacercariae. The suckers and individual organs are no larger in adult worms than in the larger metacercariae. The only increase in size results from a bulbous expansion of the posterior one-third to one-half of the body, which may be almost spherical and when bent ventrally produces a claviform appearance. The increase in size of the posterior region is caused by the development of the uterus and the accumulation of eggs in it. More than 100 eggs have been counted in a single worm. The increase in thickness and massing of eggs obscure the organs in the posterior portion of the body and they are more conspicuous in the metacercaria.

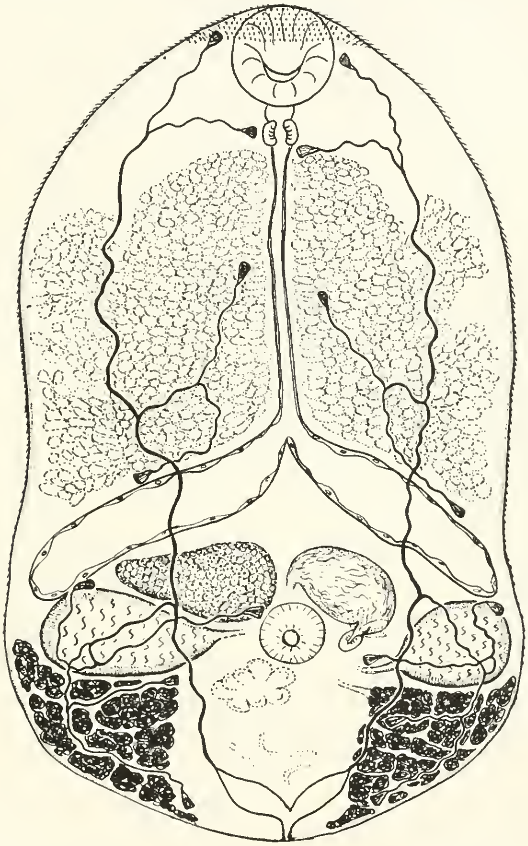

Figure 2. Fixed and stained metacercaria, 0.32 mm. long, ventral view, flattened to show excretory and genital structures.

Adult specimens, when alive and under moderate coverglass pressure, measure 0.26–0.40 mm. in length and 0.15–0.20 mm. in greatest width. The terminal egg-filled portion of the body is always wider and thicker than the flatter anterior region; otherwise the shape is unchanged from that in the metacercaria. The acetabulum measures 0.029–0.035 mm. in diameter and is located about one-third of the body length from the posterior end, a little farther forward relatively than in the metacercaria. The mouth is subterminal; the oral sucker is 0.035–0.045 mm. in diameter; the prepharynx is 0.01–0.033 mm. in length; the pharynx is 0.017–0.021 mm. in diameter; the esophagus is 0.1–0.13 mm. in length; the intestinal crura, which are preacetabular, diverge at a wide angle and measure 0.08–0.10 mm. in length. Fixed and stained specimens are somewhat smaller; the smallest gravid worm is 0.26 mm. long; 0.11 mm. in greatest width; in diameter the oral sucker is 0.025 mm., the acetabulum is 0.0225 mm., and the pharynx is 0.012 mm. The uterus of this worm contains 14 eggs.

The excretory system is unchanged from that in the metacercaria. The pore is terminal; there is a preporal sphincter, a bicornuate bladder, from which the collecting ducts pass anteriad as shown in Figure 2.

The testes are lateral, opposite, situated at least partially in the acetabular zone and about midway between the anterior and posterior ends of the enlarged portion of the body; they are oval to ovate, 0.05–0.065 mm. in the transverse axis and 0.03–0.04 mm. in the longitudinal one. Sperm ducts arise from the median faces, pass mediad and anteriad, join above the acetabulum, and the common duct expands into a large, transverse, oval seminal vesicle which may be as large as one of the testes and is located medially or slightly to the left in the area directly behind the digestive ceca. From the vesicle, an ejaculatory duct curves posteriad, mediad, and ventrad. It terminates in the male papilla which opens into the genital atrium near the left margin of the acetabulum (Fig. 3). The distal portion of the seminal vesicle and the ejaculatory duct are enclosed in glandular cells. The male papilla is very small, 0.006–0.0075 mm. in diameter and 0.012–0.014 mm. long.

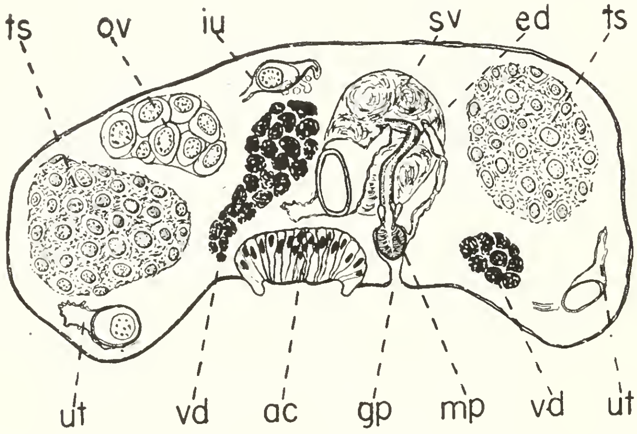

FIGURE 3. Composite drawing of two adjacent, slightly oblique, 10 μ sections, to show relations of acetabulum, gonads, genital pore, male papilla, vitelline ducts (one of which is enlarging before joining with the one from the opposite side), and dorsal to it the initial coil of the uterus containing a newly formed egg; ac, acetabulum; ed, ejaculatory duct; gp, genital pore; iu, initial coil of uterus; mp, male papilla; ov, ovary; sv, seminal vesicle; ts, testis; ut, uterus; vd, vitelline duct. Parenchyma not shown.

The ovary is oval to triangular, longer transversely, 0.06–0.065 by 0.036–0.045 mm., situated in the area bounded by the right cecum, the right testis, the acetabulum and the seminal vesicle. The details of the female reproductive ducts were observed in serial sections. The oviduct arises from the postero-median face of the ovary and coils posteriad and ventrad; there is a small enlargement which contains spermatozoa and from which Laurer's canal winds dorsad to open near the midline, just behind the level of the acetabulum. Shortly after the origin of Laurer's canal, the oviduct turns dorsad and anteriad, receives a short common vitelline duct, and expands into the ootype. Mehlis' gland consists of only a few cells. The vitellaria are large, lobed glands, situated on either side, filling the regions between the testes and the posterior wall of the body. Ducts from the two sides pass mediad and anteriad; they unite to form the common vitelline duct which opens into the oviduct as noted above. The initial portion of the uterus is dorsal to the acetabulum (Fig. 3). The uterus passes posteriad in a sinuous course near the median plane almost to the end of the body, then coils anteriad, ventrally, along the right side of the body to the level of the cecum, turns posteriad and passes backward mediad to the ascending limb; near the posterior end of the body the uterus crosses to the left side of the body where it forms a loop similar to the one on the right side and the recurrent limb turns mediad to open into the genital atrium. The course of the uterus becomes more coiled and irregular as it is filled with eggs. The eggs are oval to ovate,

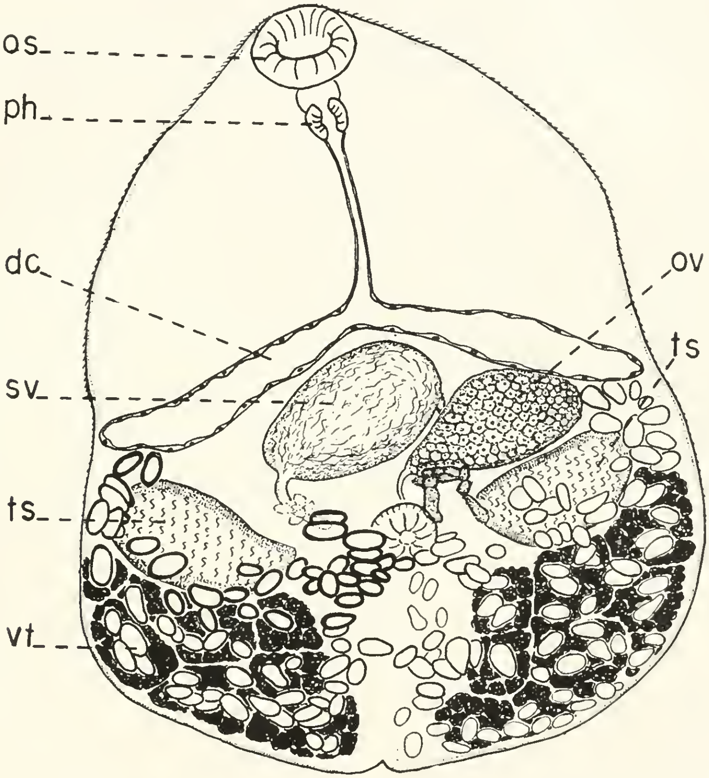

FIGURE 4. Fixed and stained specimen, 0.295 mm. long, with 120 eggs in the uterus, dorsal view, flattened to show details of the reproductive organs; dc, digestive cecum; os, oral sucker; ov, ovary; ph, pharynx; sv, seminal vesicle; ts, testis; vt, vitellaria.

operculate, 0.016–0.02 by 0.009–0.011 mm., and some of them have a very small knob at the antopercular end. Eggs in the initial portion of the uterus are almost colorless; those on the left side of the body are bright yellow. Mature eggs contain ciliated miracidia. In the metacercaria, the vitelline follicles form a compact triangular mass, extending on each side from the testis to the posterior end of the body; in gravid specimens the vitellaria and testes are enclosed by coils of the uterus, which in part obscure their outlines.

Second intermediate host: *Limulus polyphemus.*

Definitive hosts: experimentally, white mice and golden hamsters.

First intermediate host: unknown.

Type and paratype specimens: deposited in the Helminthological Collection of the U. S. National Museum, slide No. 47592.

Discussion

The taxonomic position of the species described in the present paper is obscured by certain peculiar difficulties. The asexual stages, and the natural hosts of the adult are yet unknown. Development in mice and hamsters may have produced specimens substantially different from those maturing in normal hosts. The present specimens undoubtedly belong to the genus *Microphallus,* but the generic concept is still somewhat tentative and many of the specific descriptions are incomplete. The genus *Microphallus* was erected by Ward (1901) to contain a species described by him (1894) as *Distoma opacum.* The worms were found in fresh-water fishes: *Amia calva, Ictalurus punctatus,* and *Perca flavescens* from Lake St. Clair, Michigan. Metacercarial stages were encysted in crayfish, *Cambarus propinquus.* Stossich (1899) erected the genus *Levinsenia* and included *D. opacum* in it, but Looss (1899) argued that *D. opacum* Ward did not belong in *Levinsenia* and that probably it represented a genus as yet unnamed. Ward (1901) defined the new genus *Microphallus* and proposed a new subfamily, Microphallinae, to contain it and *Levinseniella.* The subfamily Microphallinae was included in the family Heterophyidae Odhner, 1914 by several subsequent authors, including Fuhrmann (1928), although Travassos (1921) had removed the subfamily from the Heterophyidae and elevated it to family status. The more recent morphological and life-history studies of Rankin (1939a, 1939b, 1940a, 1940b), of Cable and Hunninen (1940), Baer (1943) and other authors have established the Microphallidae as a family distinct from Heterophyidae.

As noted, Stossich (1899) erected the genus *Levinsenia* to contain four species: *Distomum brachysomum* Creplin, 1837; *D. macrophallus* v. Linstow, 1875; *D. pygmaeum* Levinsen, 1881; and *D. opacum* Ward, 1894. In the same year, Lühe and Looss, independently, designated *L. brachysoma* as type of *Levinsenia.* The next year, Jägerskiöld (1900) proposed *L. pygmaeum* as type of the genus. Ward (1901) designated *D. opacum* as type of a new genus *Microphallus;* he stated that *Levinsenia* Stossich was preoccupied (*Levinsenia* Mesnil, 1897) and that Stiles and Hassall were to propose the name *Levinseniella* to replace it. Although the paper by Stiles and Hassall did not appear until 1902, the name *Levinseniella* was validated by Ward (1901) with *Levinseniella brachysoma* as type species. Jägerskiöld (1901), although aware of the announcement by Ward, proposed the name *Spelotrema* for the invalid name *Levinsenia,* with *S. pygmaeum* as type. He stated, p.

982, "Ich sehe aber aus Ward's Aufsatz: On the Structure of the Copulatory Organs of *Microphallus* n. g. (Studies from the Zoological Labor. The Univ. of Nebraska), dass Stiles mir zuvorgekommen ist, und lasse daher, obgleich ich den Aufsatz von Stiles noch nicht habe finden können, seinen Namen *Levinseniella* gelten." Jägerskiöld (1904) listed *Levinseniella* as a synonym of *Spelotrema* although he (1907) recognized the two as distinct genera. Jägerskiöld (1901) proposed *Spelotrema* as a replacement for *Levinsenia* and regarded the two as identical, but actually this is not the case since *L. brachysoma* was designated as type of *Levinseniella* and *L. pygmaeum* as type of *Spelotrema*, and the two species are not congeneric. The name *Spelotrema* has had an unsavory reputation and the history of the name and of the generic concept were reviewed by Cable and Hunninen (1940). They discussed the nomenclatorial problem outlined above. However, the distinctness of the species allocated to *Spelotrema* and those included in *Levinseniella* was demonstrated by the studies of Rankin previously cited.

The problem of *Spelotrema* appears to be solved. Baer (1943) stated that there are no morphological differences which distinguish *Spelotrema* from *Microphallus*, and that the two genera had been maintained because species of *Spelotrema* are typically parasites of birds whereas those of *Microphallus* occur in fishes and amphibians. Baer argued that difference in host is not a proper criterion for generic distinction, since the metacercariae of these species are encysted in crustaceans which are eaten by both birds and fishes. The metacercariae are well developed and become sexually mature in a few days after ingestion by the definitive hosts. Moreover, certain species mature in mammals as well as birds and host-specificity is not at all precise. Since *Microphallus* antedates *Spelotrema*, the latter name was suppressed as a synonym and the species formerly included in *Spelotrema* were transferred to *Microphallus*. The identity of *Microphallus* and *Spelotrema* has been accepted by Rausch (1947), Rausch and Locker (1951) and by Cable and Kuns (1951).

The number of valid species in the genus *Microphallus* is yet uncertain. Osborn (1919) described *M. ovatus* from *Micropterus dolomieu* taken in Lake Chautauqua, New York. Strandine (1943) compared specimens of *Microphallus* from *Amia calva* and *Micropterus dolomieu* of Lake Lelanau, Michigan, and found no significant difference between *M. opacus* and *M. ovatus*. He reported overlapping variations in total size, size of acetabulum, extent of spination, length of ceca, and size of eggs in the two previously accepted species. Rausch (1946a, 1946b) discussed morphological variation and host-specificity of species in the genus *Microphallus* and reported natural infections by *M. ovatus* in three species of turtles and in the racoon. He (1947) found that microphallid cysts from crayfish fell into two distinct size groups, the larger of which (1.3 by 0.9 mm.) agrees with the measurements given by Ward (1894) for cysts of *M. opacus*. Although the cysts were of two sizes, even from the same crayfish, the metacercariae were reported to be morphologically identical and all were able to mature when fed to suitable experimental hosts. He was unable to excyst the larvae by mechanical means, but found that they emerged in 48 hours when placed in Ringer's solution with fragments of the gland in which they were found. On release, they began to copulate and when left in normal saline, Ringer's solution, or media devised for culture of protozoa, they matured and produced large numbers of apparently normal eggs. He fed metacercariae to various kinds of possible hosts; the cysts failed to hatch in chicks and toads; the metacercariae emerged but were voided in an immature condition by certain snakes. Sexually mature worms were recovered from two species of turtles, two snakes, the opossum and the racoon, but only negative results were obtained with salamanders, frogs, chicks, albino rats and a skunk. Adults from the two types of cysts differed in size, but since they were otherwise so similar, Rausch concluded that they belong to a single species. Furthermore, he suggested that *M. gracilis* Baer, 1943, may also be identical with *M. opacus.* These experiments of Rausch are of fundamental significance, since they exemplify the surest method of determining specific relations among parasitic worms. His data suggest that there are actually two species represented in his material, and *M. ovatus* may be distinct from *M. opacus.*

Diagnoses of previously described species in the genus *Microphallus* (syn. *Spelotrema*) were made by Rankin (1940a), Cable and Hunninen (1940), Baer (1943) and Rausch and Locker (1951). Earlier authors, Odhner (1905), Nicoll (1907), and more recent ones, Rankin, Cable and Hunninen, and Baer, regarded the size of the male papilla as a diagnostic, specific feature, whereas Rausch and Locker emphasized the length of the digestive ceca and the relative size of suckers. Rankin (1940a) described the life-cycle of *Spelotrema papillorobusta* n. sp., and listed the specific details of five previously described species. Cable and Hunninen (1940) tabulated the specific features of five species which they compared with *Spelotrema nicolli,* a species described by them as new and for which they traced the life-history. Baer (1943) described *M. gracilis* n. sp., from *Neomys fodiens,* suppressed *Spelotrema* as a synonym of *Microphallus,* and recognized fourteen species, thirteen of which were distinguished in a key. The fourteenth species, *M. minus* Ochi, 1928, was not included as the description was published in Japanese. The genus *Monocaecum* Stafford, 1903, was suppressed as a synonym of *Microphallus* and its only species, *M. baryurus* was listed as possibly identical with *M. ovatus.* Baer noted that the metacercariae attain almost complete maturity in their crustacean intermediate hosts, that they become gravid in a few hours in the anterior portion of the intestine of various cold-blooded and warm-blooded hosts, and that they diminish in numbers from day to day as they migrate toward the posterior portion of the intestine. To account for these facts, he suggested that the metacercariae have developed a "progenèse retardée" or "néoténie naissante," and that the cold-blooded hosts may be "hôtes d'attente" to which the parasites have become adapted. Rausch and Locker (1951) described *M. enhydrae* n. sp., from the sea-otter, *Enhydra lutris,* and recognized fourteen species in the genus *Microphallus.*

The present specimens agree most closely with the description of *M. claviformis* (Brandes, 1888), described originally from the rectum of *Tringa alpina* at Halle, Germany. Brandes stated that the body was often constricted into two regions, with a flattened anterior portion about twice as long as a swollen posterior portion, which gave the worms a club-shape and suggested the specific name, *Distomum claviforme.* The description and figure give an incomplete, inadequate, and probably erroneous characterization of the species. Lühe (1899) stated, "Bei *Dist. claviforme* Brds., welches sich durch die auffällige Länge von Praepharynx und Oesophagus auszeichnet, ist die Lage und Form der Dotterstöcke und die Lage des Genitalporus vollkommen unbekannt, so dass die Art meines Erachtens zur Zeit in ein System nicht eingereiht werden kann." Jägerskiöld (1900) added, "Was aber *Distomum claviforme* Brandes betrifft, so bin ich, mit der Ansicht Lühe's anschliessend, eher

geneigt zu glauben, dass es zu den nie zu identifizierenden Arten gehört, wenn es nicht etwa dem Autor selbst gelingen wird, es wiederzufinden." Jägerskiöld suspected, and justifiably, that Brandes had misidentified the internal organization, that the reported anterior testis was the seminal vesicle, that the posterior testis was the cirrus sac or gonotyl, and that the actual testes and vitellaria were obscured by the windings of the uterus. Brandes suggested that the worms might be the adult stage of a metacercaria encysted in the crab, *Carcinus maenas*.

What was presumed to be the same species was redescribed by Nicoll (1907) as *Spelotrema claviforme* from *Pelidna* (*Tringa*) *alpina* and *Aegialis histicula*. He noted that the sexually mature worms were smaller than the metacercariae encysted in *C. maenas*, thus nullifying the suggestion of Brandes. In a second paper, Nicoll (1909) restated the specific diagnosis, included a figure, and added *Anthus obscurus*, *Numenius arquata*, *Moticilla flava* and *Larus ridibundus* as new hosts of the parasite. Apparently Brandes and Nicoll are the only authors who have published original observations of the species, although it had been transferred to *Brachycoelium* by Stossich (1892) and to *Lecithodendrium* by Stossich (1899).

The only morphological feature in which the present specimens differ from the description of Nicoll is in the size of the male papilla, which is only about one-half the size of that in the specimens described by Nicoll. The worms from *Limulus* may indeed be specifically identical with those described by Nicoll, and at first they were referred to *M. claviformis*. But bionomic features seem to preclude the allocation of the specimens to that species. In all other microphallids for which the life-history is known, the metacercariae occur in crustaceans. While larvae of this species may encyst in crustaceans, the intense infection in *Limulus* suggests that the horseshoe crab is the natural host, and *Limulus polyphemus*, a living representative of the Xiphisurida, is a chelicerate form related more closely to the pycnogonids and the scorpions than to the crustaceans. It is far removed, zoologically and phylogenetically, from the Crustacea. Moreover, *Limulus* does not occur on the coast of Europe and some other animal must serve there as the second intermediate host of *M. claviformis*. Adult stages of the present species have so far been recovered only from mammals; and possible bird hosts, other than the chick, have not been tested. If the species is actually *M. claviformis*, it should mature in *Larus ridibundus* when young birds are available. Presumably, older and previously infected birds would prove refractory.

Other species of *Microphallus* reported from mammalian hosts include *M. minus* by Ochi (1928) from rats, cats, dogs and man in Japan; *M. brevicaeca* by Africa and Garcia (1935) from man in the Philippine Islands; *M. gracilis* by Baer (1943) from *Neomys fodiens* taken in the environs of Geneva, Switzerland; *M. opacus* by Rausch (1947) from the opossum and the racoon; and *M. enhydrae* by Rausch and Locker (1951) from the arctic sea-otter. The metacercariae of *M. minus* and *M. opacus* occur in crustaceans and *M. opacus* matured in both poikilothermal and homothermal hosts. Since Rausch has shown that *M. opacus* can mature in opossums and racoons, the fact that the present specimens developed in mammals may be incidental and insignificant. *M. opacus*, like the present species, failed to infect chicks. It is entirely probable that the metacercariae in *Limulus* may mature in shore-birds. When the asexual stages and cercariae of the species are discovered, it will be possible to attempt infection of crustaceans and learn whether or not the parasite can use both arachnids and crustaceans as second intermediate hosts. Since the microphallid cercariae are very immature and the metacercariae undergo extensive development in the second intermediate host, it must be more than a mere transfer host. In the present state of uncertainty concerning specificity in the genus *Microphallus* it seems inappropriate to assign the specimens described in this paper to *M. claviformis*, and they are designated as *M. limuli* n. sp. Actually the features employed by previous authors to distinguish between species of *Microphallus* are exceedingly variable and tenuous; and certain of the presently accepted species are probably identical. The experimental results of Rausch (1947) have thrown doubt on the validity of several species and, in correspondence, Cable has recently suggested that *M. nicolli* may be the same as *M. simile*.

In the paper mentioned earlier, Baer (1943) divided the family Microphallidae Travassos, 1921, into two families, Microphallidae and Maritrematidae, distinguished by the absence of a cirrus sac in the former and the presence of this organ in the latter. In the family Microphallidae, as restricted, he included the genera: *Microphallus* Ward, 1901; *Levinseniella* Stiles and Hassall in Ward, 1901; *Spelotrema* Jägerskiöld, 1901; *Monocaecum* Stafford, 1903; and *Spelophallus* Jägerskiöld, 1908. The family Maritrematidae, as conceived, contained the genera: *Maritrema* Nicoll, 1907; *Microphalloides* Yoshida, 1938; *Gynaecotyla* Yamaguti, 1939 (misspelled *Gynaecocotyla*,= *Cornucopula* Rankin, 1939); and *Pseudospelotrema* Yamaguti, 1939 (= *Maritreminoides* Rankin, 1939). It is to be noted that although Baer included five genera in the restricted family Microphallidae, in the same paper he suppressed *Spelotrema* and *Monocaecum* as identical with *Microphallus*. Moreover, he noted that *Spelophallus* differs from *Spelotrema* in only a single feature; that in the former genus the metraterm opens into the distal part of the genital atrium whereas in the latter it opens deeper in the atrium, near the base of the male papilla. *Spelophallus* is known by only a single species, *S. primus* Jägerskiöld, 1908. In these small, soft-bodied worms, where lack of skeletal support and contraction of different sets of muscles can produce extensive variation in the relations of such mobile parts, it is questionable whether *Spelophallus* has any true morphological basis. In my opinion, the differences described by Jägerskiöld can have no more than specific value. Accordingly, *Spelophallus* is suppressed as a generic concept and the species, *S. primus*, is transferred to *Microphallus* as *M. primus* (Jägerskiöld, 1908).

The proposal of Baer to divide the family Microphallidae was analyzed by Cable and Kuns (1951). They erected a new genus, *Carneophallus*, to contain *C. trilobatus* n. sp., and recognized a series of genera from *Microphalloides* and *Pseudospelotrema* to *Levinseniella* and *Carneophallus*, showing gradual reduction of the cirrus sac correlated with increased complexity in structure of the copulatory organs. Accordingly, they rejected the proposal of Baer, retained the genera with a cirrus sac in the family Microphallidae, and presented a scheme to illustrate probable evolution in the family. The argument of Cable and Kuns seems convincing and there appears to be no adequate basis for recognition of a family Maritrematidae.

Summary

Metacercariae from *Limulus polyphemus* developed to sexual maturity in white mice and golden hamsters. The worms are described as a new species, *Microphallus limuli*. Morphologically they are very similar to *M. claviformis* (Brandes, 1888), but bionomic features seem to preclude their allocation to that species. The genus

Microphallus is discussed, *Spelophallus* Jägerskiöld, 1908 is suppressed as a synonym and *S. primus* Jägerskiöld, 1908, the only known species, is transferred to *Microphallus* as *M. primus* (Jägerskiöld, 1908). In agreement with Cable and Kuns (1951), the family Maritrematidae Baer, 1943 is not accepted.

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NEW SPECIES OF SYNDESMIS AND A REVISION OF THE FAMILY UMAGILLIDAE WAHL, 1910 (TURBELLARIA: RHABDOCOELA)

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During the spring and summer of 1948 at the Scripps Institution of Oceanography, La Jolla, California, one of us (H. W. S.) examined various marine invertebrates for possible stages in the life-history of the mesozoan parasites of octopuses.[2] In dissections of the common sand-dollar, *Dendraster eccentricus,* several specimens of a rhabdocoel turbellarian were found. Whether they were originally in the intestine or the perivisceral cavity was not determined. Two specimens were fixed in Duboscq-Brasil solution, stained with Paracarmine, and mounted. Attempts at identification showed them to be different from all previously described species. Accordingly, preliminary sketches were made and the larger one was demounted, cut transversely in serial sections at 10 microns, and stained with haematoxylin and erythrosin. The presence of a large egg-capsule in each worm obscured certain structures in the whole mounts, and in the sectioned specimen, the adjacent structures were obviously distorted by pressure.

So far as we have been able to determine, this is the first rhabdocoel parasite to be described from the flattened, sand-dollar type of echinoid and the fifth species of the family Umagillidae reported from the Western Hemisphere. The morphology of the worms and review of the literature indicated their inclusion in the genus *Syndesmis.* Since the specimens could not be referred to any known species, they are described as a new species for which the name *Syndesmis dendrastrorum* is proposed.

THE GENUS SYNDESMIS

The genus *Syndesmis* was erected by Silliman (1881) but without specific designation, for a parasite of *Echinus sphaera* found at Roscoff, France. The account, without figures, was adequate to validate the generic name. What was probably the same species had been reported, but not named, by Geddes (1880) from the perivisceral fluid of the same species of sea-urchins at Roscoff. François (1886) reported *Syndesmis* from the intestine of *Strongylocentrotus lividus* and *Echinus acutus* at Banyuls sur Mer in the Mediterranean, emended the description of Silliman (misspelled Sillimann), and proposed the specific name, *S. echinorum.* Cuénot (1892) found *S. echinorum,* at least a score in each individual, in the coelomic cavity of *Echina sphaera* at Roscoff; he described the activities of the worms and stated that,

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like many other animals which live in ecological niches where the CO_2 is high and the O_2 tension is low, they contain haemoglobin in the lacunae of the mesenchyme. Russo (1895) gave a more complete account, illustrated with 36 figures, of the morphology of *S. echinorum* from *Sphaerechinus granularis* at Naples, with observations on the development and homologies of the genital structures. He stated that the parasites were located in the digestive tract rather than the coelom. Shipley (1901) described *S. echinorum* (misspelled *Syndesmus*) from ten specimens found in the alimentary canal and one from the perivisceral cavity of *Echinus esculentus* at Plymouth, England. He noted that the earlier accounts by Silliman, François, and Cuénot had promised complete descriptions, which apparently were never published. Shipley was aware of Russo's paper, but had not seen it. Von Graff (1903) stated that the genus *Syndesmis* contains a single, widely distributed species which he had found in the intestine of *Sphaerechinus granularis* at Bergen and of *Strongylocentrotus dröbacchensis* at Alexandrowsk. He described the characteristic movements of the worm and discussed the homologies of the female genital ducts, for which different terminology had been employed by previous authors.

Subsequent accounts have been given by other authors. Briot (1906) described, without figures, specimens which he found in the perivisceral cavity and intestine of *Strongylocentrotus lividus* and *Sphaerechinus granularis* at Marseilles, France. The worms were much larger than those reported previously and the measurements may have been made from much flattened specimens. He observed the exudation of red material from the mouths of pressed specimens and predicated that the pigment in the parasites came from the tissues of their hosts. Differences between worms from the two species of sea-urchins led to the belief that two distinct varieties were involved. In a series of papers, Wahl (1906, 1909, 1910a, 1910b) made important contributions to knowledge of the morphology and taxonomy of *Syndesmis*, although his work was overlooked by a number of later investigators. The monograph by von Graff (1913) on the rhabdocoel turbellarians listed *Syndesmis* with *S. echinorum* as the only species. Meixner (1923) discussed the origin and function of the egg-capsules and polar filaments of *Syndesmis* and other rhabdocoels. Westblad (1926a) described the excretory system of *S. echinorum*. Although most workers have regarded the syndesmid rhabdocoels of European sea-urchins as representatives of a single species, the accounts of the several authors list many differences in host, habitat, size, and internal structure. The figures of *S. echinorum* by Russo and by Shipley (Figs. 1, 2) show many differences, especially in shape and location of the testes and in the length and course of the genital ducts; consequently there appears some question whether *S. echinorum* designates a single species or a group of related forms. Indeed, Braun (1889) suggested that the descriptions of Silliman (1881) and François (1886) were concerned with different species.

A second species of *Syndesmis* was reported but not named by Powers (1936). About five per cent of the sea-urchins, *Centrechinus antillarum*, examined at the

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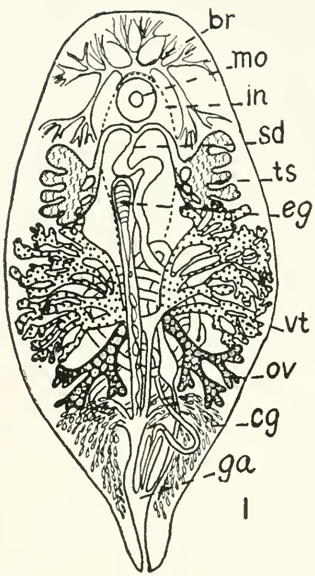
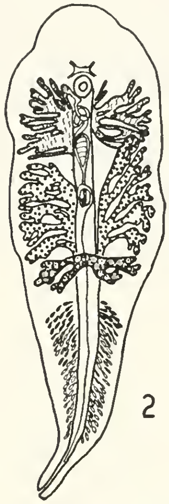
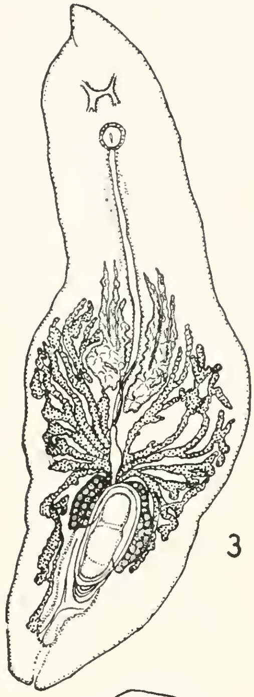
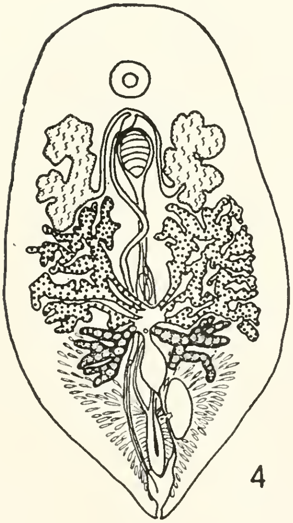
The figures are copied or redrawn from original descriptions with special emphasis on the shape, size, location and relations of the testes, ovaries, and vitellaria, which we regard as features of essential taxonomic significance. Structures are similarly represented in all species and labelled in Figure 1. Abbreviations: br, brain; cg, cement glands; eg, egg-capsule in uterus; ga, common genital antrum; in, intestine; mo, mouth; ov, ovary; sd, common sperm duct; ts, testis; vt, vitellaria.

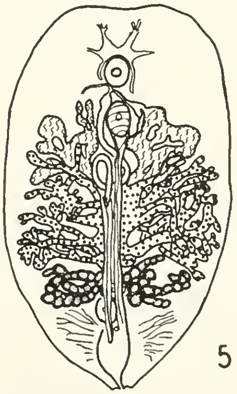
PLATE I











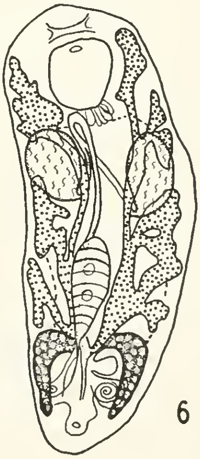


FIGURE 1. *Syndesmis echinorum* after Russo, 1895.
FIGURE 2. *Syndesmis echinorum* after Shipley, 1901.
FIGURE 3. *Syndesmis dendrastrorum* new species, original, camera lucida.
FIGURE 4. *Syndesmis franciscana* (Lehman, 1946) after Lehman.
FIGURE 5. *Syndesmis antillarum* new species, after Powers, 1936.
FIGURE 6. *Umagilla forskalensis* after Wahl, 1906.

Tortugas Laboratory, off the Florida Coast, were infected, each with five to sixteen worms in the coelomic cavity, attached to the outer wall of the intestine. The parasites were bright red in color, 1–1.5 mm. long, and according to Powers, related to *S. echinorum.* The specimens were compared with Shipley's description of *S. echinorum*, from which they were reported to differ in the arrangement of the cement glands, in the general morphology of the posterior region, and in details of the copulatory apparatus. The reported differences were portrayed in a text-figure. Powers labelled the figure "*Syndesmus* sp.," unfortunately adopting Shipley's misspelling of the generic name. The scale at the side of the figure indicates that the specimen figured was about 2 mm. long. Powers' account and figure validate his findings and for the species we propose the name *Syndesmis antillarum,* with the specific description derived from Powers' statement and figure. In the description we have employed the revised terminology of Dr. L. H. Hyman to designate the reproductive organs of the Turbellaria, rather than the original terminology of Powers.

Syndesmis antillarum new species (Fig. 5)

Living specimens bright red in color, 1–1.5 mm. long, oval to elongate, rounded or flattened anteriorly, somewhat attenuate posteriorly. Pharynx located in the anterior fourth of body, immediately posterior to the commissure of the nervous system. Testes paired, lobed to dendritic, in anterior half of body, but postpharyngeal. Muscular base of penis near middle of body, penis extends about one-third of body length. Vitellaria dendritic, with several (5–8) main stems on each side, located between the testes and ovaries and partially overlapping the testes. Ovaries paired, lobed or branched, located in posterior third of body, immediately posterior to the vitellaria. Uterus in anterior half of body; single egg-capsule with long filament; cement glands surround the genital antrum; bursal canal short; seminal bursa oval, immediately posterior to the oval, seminal receptacle. Habitat: coelom of the sea-urchin, *Centrechinus antillarum*, Dry Tortugas, Gulf of Mexico.

Syndesmis franciscana (Lehman, 1946) (Fig. 4)

Lehman (1946) described a parasitic turbellarian from the California sea-urchin, *Strongylocentrotus franciscanus* as *Syndisyrinx franciscanus.* He noted that *Syndesmis* and *Syndisyrinx* are parasites of echinoids and that in morphological features they are very similar, which suggested close relationship between the two genera. *Syndisyrinx* was distinguished chiefly on the structure and relations of the seminal bursa which is connected by cuticular ducts to the seminal receptacle and bursal canal. The penis was described as a cuticular hollow stylet. The name, *Syndisyrinx,* was chosen to indicate the presence of a "bursal valve," a cuticular structure seven microns in diameter and ten microns long, composed of the ends of the bursal and insemination canals and the sheath which surrounds them. These cuticular canals have been described for other members of the family although a minute sheath around their openings has not been reported. Lehman compared his specimens with the description of *Syndesmis echinorum* given by Russo (1895). On the basis of Meixner's (1926) re-description of that species, not cited by Lehman, Marcus (1949) suppressed *Syndisyrinx* as a synonym of *Syndesmis.* We concur in the opinion of Marcus and designate the species described by Lehman as *Syndesmis franciscana* (Lehman, 1946).

Syndesmis dendrastrorum new species (Fig. 3)

The turbellarian from sand-dollars of the Pacific coast is distinct from *S. echinorum* as described by the several European authors, from *S. antillarum* as figured by Powers, and from *S. franciscana* (Lehman, 1946). The description is based on laboratory notes and on two specimens, as noted earlier.

Living specimens colorless to gray, elongate oval to fusiform, flattened ventrally and slightly convex dorsally, widest at or just posterior to the middle of the body, 1–2.5 mm. in length, capable of much elongation with accompanying decrease in width. Two worms, flattened under one-half of a glass slide, fixed, stained and mounted, measured 1.58 by 0.42 mm. and 1.14 by 0.36 mm., respectively. Both were mature, each with a large egg-capsule in the uterus. The smaller specimen is shown in Figure 3. In it the egg-capsule measures 0.105 by 0.072 mm.; the capsule in the larger worm measured 0.198 by 0.08 mm. This worm was sectioned serially. The body is entirely covered with cilia, which measure about 3 microns in length. The epithelium is 4–6 microns in thickness, the cells roughly hexagonal in surface view, but cell boundaries are often indistinct. The nuclei measure about 4 microns in diameter and are often somewhat flattened. The cells rest upon a basement membrane and below it there is a thin layer of circular fibers, on the inner side of which there are small, separated, longitudinal fibers. The parenchyma is loose and vacuolate, traversed by many dorso-ventral fibers. The pharynx, of typical "doliiformis" structure, is situated in the anterior fourth of the body and measures about 50 microns in diameter. The intestine is narrow, tubular, dorsal in position; its walls consist of irregularly-shaped cells. It has no diverticula, does not extend anterior to the pharynx, and ends posteriorly a short distance anterior to the egg-capsule (about 45 microns in the sectioned specimen). The nerve commissure is located about two-thirds of the distance from the anterior end of the body to the pharynx.

The testes are paired, lobed to dendritic, in the posterior part of the anterior half of the body, a considerable distance posterior to the pharynx. The origins of the sperm ducts were not observed. The common sperm duct was traced forward from the base of the "penis" for a short distance between the posterior ends of the testes but could not be followed to its bifurcation into the sperm ducts. A small, more densely staining area, situated about two-thirds of the distance from the testes to the pharynx may represent a spermiducal vesicle. The muscular base of the penis is situated about three-fifths of the body length from the anterior end; the copulatory organ is a small, narrow, cuticular tube (about 2 microns in diameter), ventral in position, extending posteriad to open near the posterior end of the genital antrum. Vitellaria paired, dendritic glands, located principally in the middle third of the body; branches extend forward and laterad to the level of the middle of the testes, and backward, lateral to the ovaries, to the level of the genital antrum. The branches converge anterior to the ovaries; union of vitelline ducts with seminal receptacle not observed. Ovaries paired, lobed or branched, located in the posterior third of body, partially obscured by the large egg-capsule in the uterus. Egg-capsule large, in posterior third of body, filament relatively short (less than twice capsule length), twisted and tangled. Sparse unicellular cement glands in the posterior fourth of body. Seminal bursa oval, immediately posterior to and confluent with oval seminal receptacle; bursal canal narrow, long; ovovitelline duct short; genital antrum opens at posterior end of body. The large egg-capsule exerted pressure on and distorted adjacent structures, preventing detailed study of the genital antrum and the ducts which open into it.

From the features listed, it appears proper to refer the specimens to the genus *Syndesmis.* They differ from *S. echinorum, S. antillarum* and *S. franciscana* in the greater distance between the pharynx and testes, in form of the intestine, greater extent of vitellaria, smaller pharynx, more posterior location of the uterus, sparser cement glands, larger size of egg-capsule and shorter and more delicate capsular filament. Its host belongs to a different order of the Echinoidea. These differences may be regarded as characteristic of *S. dendrastrorum.* The type specimen has been deposited in the U. S. National Museum, U.S.N.M. Helm. Coll. No. 47593.

The Family Umagillidae

Von Graff (1882) erected the family Vorticidae to contain certain rhabdocoel turbellarians and provisionally separated the parasitic from the free-living species. In a revision he (1903) dropped the subfamily divisions and included the parasitic genera, *Anoplodium* Schneider, 1858, *Syndesmis* Silliman, 1881, and *Collastoma* Dörler, 1900, with several free-living genera in the family Vorticidae. With the acceptance of *Dalyellia* as the valid generic name for the species formerly included in *Vortex,* he (1905) proposed the family name Dalyelliidae with two subfamilies: Dalyelliinae with paired ovaries and Graffillinae with unpaired ovary; the latter he (1908) raised to family rank. The genus *Anoplodium* was included in the family Dalyelliidae; the genera *Syndesmis, Collastoma* and *Umagilla* in the family Graffillidae.

Wahl (1906) published the first of a series of papers on the parasitic turbellarians. He studied the morphology of members of the genera *Anoplodium, Graffilla, Paravortex, Collastoma,* and erected a new genus, *Umagilla.* Wahl (1909) published further studies on *Umagilla* and *Syndesmis,* and showed that the basis of von Graff's (1905) arrangement was untenable. He (1910a) presented further evidence, based especially on the female genital ducts, to show that *Umagilla, Syndesmis, Anoplodium* and *Collastoma* constitute a related group, distinct from the other genera included in von Graff's families Dalyelliidae and Graffillidae. The genus *Umagilla* was regarded as the most primitive and characteristic member of this group and was named type of a new family, Umagillidae. In the family, "Die als Receptaculum seminis fungierende Vereinigungsstelle von Keim- und Dotterstöcken (bezw. von deren Ausführungsgängen) ist mit dem Atrium genitale durch einen Ductus communis und eine Vagina verbunden." Two subfamilies were recognized: Umagillinae with paired testes and Collastominae with a single, median testis. The first subfamily included *Umagilla, Syndesmis* and *Anoplodium;* the second only the genus *Collastoma.* Wahl (1910b) made further studies on the genus *Collastoma.*

Von Graff (1913) accepted the systematic arrangement proposed by Wahl (1910a), in which the family Graffillidae was suppressed and the genera, other than those included in the Umagillidae, were transferred to the family Dalyelliidae. However, without explanation and in contravention of the Rules of Nomenclature, he proposed the family Anoplodiidae and the subfamily Anoplodiinae, listing Umagillidae and Umagillinae as synonyms. Six valid and five doubtful species were included in the family.

Since the monograph by von Graff (1913), many additions have been made to knowledge of the family, the number of genera and species has trebled, but there has been no detailed systematic account of the group. Only four families of the Rhabdocoela are completely parasitic and the family Umagillidae is by far the largest; in fact, this family constitutes the largest parasitic group in the entire class Turbellaria. All members of the Umagillidae are parasitic, living in the intestine or body cavity of echinoderms or in one instance, in the alimentary tract of sipunculid worms. Ward (1933) noted that, "both by virtue of numbers of species parasitic in echinoderms and of frequence in those hosts the Turbellaria may rightly be designated as the major group of echinoderm parasites." Steinmann and Bresslau (1913) adopted von Graff's family and subfamily names Anoplodiidae and Anoplodiinae in their monograph on "Die Strudelwürmer". Beklemicheff (1916) erected two new genera, *Desmote* and *Pterastericola,* to contain two new species, the first from a crinoid and the second from a sea-star. These are the first parasitic turbellarians reported from their respective echinoderm hosts and both genera were included in the family Dalyellidae. In a series of papers Meixner (1923, 1924a, 1924b, 1926) discussed the morphology and classification of the rhabdocoel turbellarians; he adopted the family name Anoplodiidae and suggested a new family Pterastericolidae. Kaburaki (1925) proposed a new genus, *Bicladus,* for a new species, *B. metacrini,* from *Metacrinus rotundus.* Steinböck (1925 transferred the species to *Desmote* and suppressed the generic name *Bicladus,* but Poche (1925) accepted *Bicladus* as a valid genus and, in fact, designated it as type of a new family. Subsequent papers in which this worm has been mentioned have tacitly agreed with Steinböck's disposal of the species and have not cited Poche's paper.

Faust (1924) reported a supposed holostome trematode from the holothurian, *Actionopyga mauritiana,* from the Andaman Sea. He named the parasite *Cleistogamia holothuriana* and later (1927) published a more complete account. On the basis of Faust's description, Bosma (1931) erected a new family, Cleistogamiidae, in the trematode superfamily Strigeoidea. Influenced by Faust's (1927) paper on *Cleistogamia,* Khalil and Azim (1937) described worms from holothurians of the Red Sea as trematodes under the designation *Loutfia loutfia* n. g., n. sp. They considered *Loutfia* and *Cleistogamia* to represent a new family which, unaware of Bosma's paper, they named Cleistogamidae. Later, Khalil (1937) studied living specimens and, in a footnote to the above paper, reported that the supposed trematode was a rhabdocoel turbellarian belonging to the family Anoplodiidae. Khalil (1938) redescribed the species as *Cleistogamia loutfia.* Baer (1938) independently had studied specimens of *Cleistogamia holothuriana* deposited by Faust in the Indian Museum. He showed that Faust had confused anterior and posterior ends, misinterpreted the internal anatomy, and that a complete new description was necessary. In a postscript, Baer referred to the papers by Khalil and Azim (1937) and Khalil (1937) and pointed out that the vitellaria are so different in *Cleistogmia* and *Loutfia* that it is unlikely that the two are identical. Marcus (1949) tacitly suppressed *Loutfia* as a synonym of *Anoplodiera* in naming *L. loutfia* as *Anoplodiera loutfia* (Khalil and Azim).

Bock (1926) described a new species, *Anoplodium stichopi,* from the body cavity of *Stichopus tremulus;* for it he proposed a new subgenus, *Anoplodiella.* He mentioned, but did not describe, another new species from the same host which he referred to the genus *Umagilla.* Westblad (1926a) described the excretory system of *Syndesmis echinorum* and also (1926b) reported five species, all as members of the family Umagillidae, from *Stichopus tremulus.* One of the five, *Anoplodium stichopi,* had been described by Bock (1926) ; two others, *Anoplodiera voluta* and *Wahlia macrostylifera,* representing new genera, were described by Westblad (1930) ; the others were listed as *Umagilla elegans* Bock and *Meara stichopi* Bock. Westblad (1949) described *Meara stichopi* and showed that it is a member of the Turbellaria archoophora, suborder Nemertodermatida. Westblad (1930) discussed the female genital ducts and regarded the "female atrium" of earlier workers as a "secondary uterus"; he reported that *Umagilla elegans* may contain as many as 26 mature egg-capsules in its large "sekundären Uterus." Ozaki (1932) described *Anoplodium mediale* n. sp., from the body cavity, and *Xenometra arbora* n. g., n. sp., from the intestine of *Stichopus japonicus.* Bresslau (1933) reverted to the use of the family name Anoplodiidae, although Westblad had used the correct name Umagillidae, and the subfamilies were dropped. Eight genera and twelve species were listed and, without discussion, the subgenus *Anoplodiella* was raised to generic rank. In agreement with Steinböck (1925), the new species described by Kaburaki (1925) was listed as *Desmote metacrini.* Marcus (1949) in a monograph on the Turbellaria of Brazil, employed the invalid name Anoplodiidae on the erroneous assumption that the family name must be derived from that of the oldest genus. He noted that the name *Xenometra* Ozaki, 1932 was preoccupied by *Xenometra* Emery, 1917 and for a replacement he proposed the generic name *Ozametra.* He described a new species, *Anoplodium evelinae* and suppressed *Anoplodiella* as a synonym of *Anoplodium.*

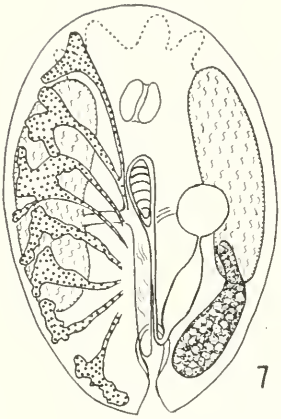
Four parasitic rhabdocoels of the family Umagillidae have been reported previously from the Western Hemisphere. The first was described by Meserve (1934) as *Macrogynium ovalis* n. g., n. sp., from *Stichopus* sp. at Bermuda. The account is inadequate, the terminology employed differs from that customarily used in describing rhabdocoels, and there is no comparison with other parasitic turbellarians. Since there is no differential diagnosis of either genus or species, as required by the action of the XIth International Congress of Zoology, the validity of the names proposed by Meserve is questionable. However, the testis and female genitalia, as described, exclude the species from other genera in the family. Marcus (1949) recognized *M. ovalis* as a valid name. The second endoparasitic rhabdocoel from the Western Hemisphere was reported by Powers (1936) as "*Syndesmus* sp." from *Centrechinus antillarum.* His account and figure provide the basis for the description in which we have named the species *Syndesmis antillarum.* The last genus to be added to the family and the third species from the Western Hemisphere was described by Lehman (1946) from the intestine of the California sea-urchin, *Strongylocentrotus franciscanus,* as *Syndisyrinx franciscanus* but, as noted earlier, Marcus (1949) suppressed the name *Syndisyrinx* as a synonym of *Syndesmis* and we have designated the species, *Syndesmis franciscana* (Lehman, 1946). The fourth species from the Western Hemisphere was described by Marcus (1949) as *Anoplodium evelinae* from the body cavity of unidentified holothurians at the Ilha da Palmas, bay of Santos and the island of São Sebastião.

In the paper mentioned earlier, Baer (1938) discussed the homology of the genital ducts in eight genera, *Desmote, Anoplodiella, Anoplodiera, Umagilla, Anoplodium, Syndesmis, Wahlia* and *Cleistogamia,* of the subfamily Umagillinae. Diagrammatic figures were presented to illustrate the relations and the genera were arranged in a series based on the length and location of the "ductus communis" (= ovovitelline duct) and the length and morphology of that portion of the female complex which intervenes between the "ductus communis" and the genital antrum. For this structure, Baer adopted the term "secondary uterus," which had been proposed by Westblad (1930). Homology of the female genital ducts is undoubtedly significant; however, relative length of ducts is not a very sound morphological character and the arrangement of genera proposed by Baer is at variance with one based on other reproductive structures as Baer himself recognized. The location and form of the testes, vitellaria and ovaries afford another and much more convenient basis for recognizing and relating the genera in the group. Which criteria will eventually prove more important in tracing interrelations between genera is yet to be determined. The morphology of all described species of the family Umagillidae is depicted in Figures 1–23, which are copied from the original descriptions with the gonads and vitellaria represented as diagnostic features.

Review of the literature on the family Umagillidae indicates the need for a re-statement of family, subfamily and generic characters. Several of the reports are incomplete, descriptions are often vague rather than precise, and any attempted revision must be regarded as tentative. It is probable that many species of the family are yet unknown and future discoveries may require extensive changes in present concepts. However, it should be helpful and perhaps stimulating to have an outline-sketch of the family as it appears at present. Accordingly we propose a revised scheme of classification.

Changes from previous arrangements are noted briefly. As stated in the abstract of the present paper (Stunkard and Corliss, 1950), we would recognize three subfamilies: Umagillinae, Collastominae, and Bicladinae. We regard *Bicladus* Kaburaki, 1925 as a valid genus and name it type of a third subfamily in the Umagillidae. It should be pointed out, however, that we do not consider the family Bicladidae Poche, 1925 as identical with the new subfamily we have proposed. Poche's family included only the single species, *Bicladus metacrini;* our subfamily includes in addition, *Desmote vorax* Beklemicheff, 1916, a species which Poche placed in the subfamily Anoplodiinae. A possible fourth subfamily is indicated. Meixner (1926), in a footnote and with no definition, proposed the family Pterastericolidae for the genus erected by Beklemicheff (1916) to contain a single species, *P. fedotovi.* It is an endoparasite of several species of sea-stars and the only turbellarian parasite reported from the Asteroidea. Beklemicheff stated that it has characteristics "de tous les Dalyelliidae en général," especially the genus *Phaenocora.* Meixner (1926) believed it more closely related to the Typhlorhynchidae and Anoplodiidae (Collastominae) than to the Graffillidae. Westblad

PLATE II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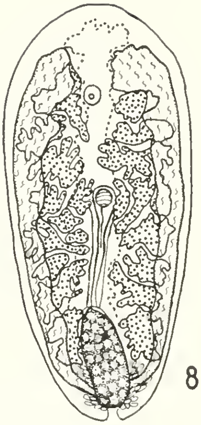
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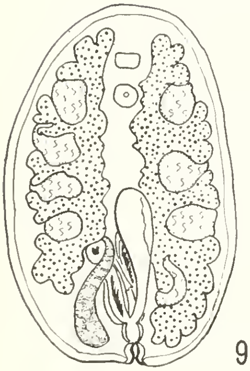
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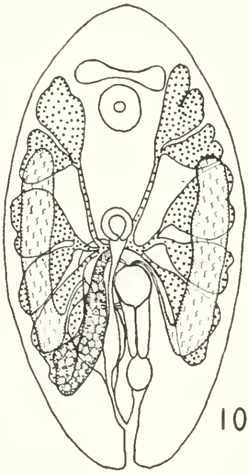
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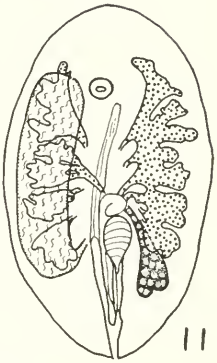
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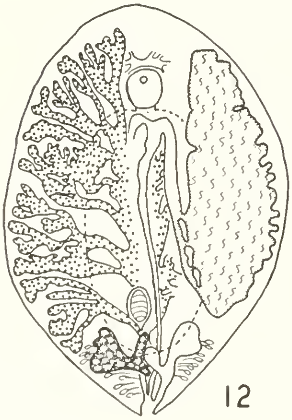
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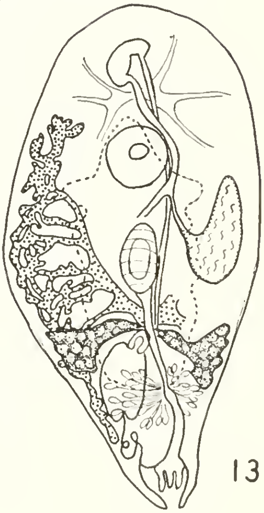
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(1926a) included the genus in the family Dalyelliidae. A diagnosis of the family Pterastericolidae was given by Bresslau (1933). The species in question (Fig. 14) is very similar to certain of the Umagillidae and it appears that the inclusion of *Pterastericola* as type of a subfamily Pterastericolinae in the Umagillidae should be considered seriously. Apparently, only the more anterior position of the genital pore contravenes the family characters listed below. In other and more minor changes, we reduce *Anoplodiella* Bock to synonymy with *Anoplodium, Syndisyrinx* Lehman to synonymy with *Syndesmis,* and pending further information, *Loutfia* Khalil and Azim is suppressed as a synonym of *Cleistogamia.*

The species of Umagillidae, recognized as valid, are arranged in a key; in addition, eight others have at one time or another been placed in the genus *Anoplodium,* either as valid or questionable species. In our opinion, they can not be recognized in the family Umagillidae. The list comprises:

1. *A. ? schneideri* Semper, 1868; the specimens were from *Stichopus variegatus* and *Mülleria lecanora* in the Philippine Islands. There was no description or figure and the name is invalid.
2. *A. ? clypeasteris* v. Graff, 1882; the name was proposed for red planarians from the surface of *Spatangus* sp., reported by Moseley (1874); Cuénot (1892) suggested a possible relationship to *Syndesmis* since both contained haemoglobin.
3. *A. ? mytili* Levinsen, 1879; an oculate species transferred to *Graffilla* by von Graff (1882).
4. *A. myriotrochi* v. Graff, 1882; reported but not described or named by Danielssen and Koren (1880) as a "smaa rødlige Planarier" from the intestine of *Myriotrochus rinkii* at Spitzbergen.
5. *A. pusillum* Monticelli, 1892; reported from the body cavity of *Holothuria poli* at Naples. Von Graff (1913) listed it as a synonym of *A. parasita.*
6. *A. graffi* ? Monticelli, 1892; from *Holothuria impatiens* at Naples; von Graff (1913) listed it as an uncertain species.
7. Vorticide (Gen. ? sp. ?) Monticelli, 1892; found in *Cucumaria planci* at Naples; no description; listed as a possible *Anoplodium* by von Graff (1903).
8. *A. chirodotae* Sabussow, 1900; from the body cavity of *Chirodota pellucida;* an oculate species which von Graff (1913) excluded from *Anoplodium.*

FAMILY UMAGILLIDAE WAHL, 1910 (SYN. ANOPLODIIDAE VON GRAFF, 1913).

Endoparasitic Rhabdocoela with no rostrum, rhabdites, dermal or adhesive glands, or eyes. Ventral "pharynx doliiformis," situated in anterior third of body; genital pore located posteriorly, single with one exception (*Desmote*). Intestine

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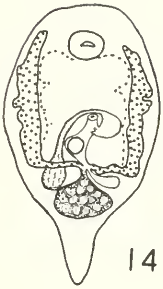
PLATE II

FIGURE 7. *Anoplodium parasita* after Schneider, 1858.
FIGURE 8. *Anoplodium mediale* after Ozaki, 1932.
FIGURE 9. *Anoplodium evelinae* after Marcus, 1949.
FIGURE 10. *Anoplodium stichopi* after Bock, 1926.
FIGURE 11. *Anoplodium gracile* after Wahl, 1906.
FIGURE 12. *Anoplodiera voluta* after Westblad, 1930.
FIGURE 13. *Wahlia macrostylifera* after Westblad, 1930.

PLATE III



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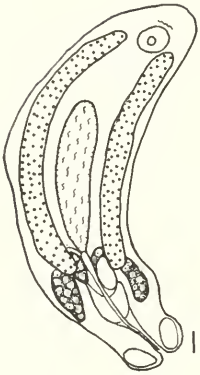

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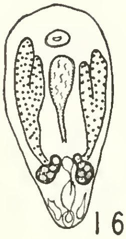

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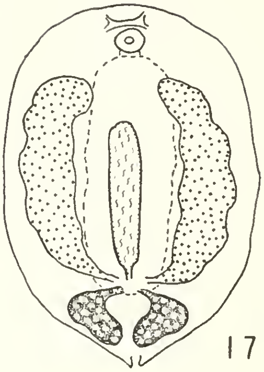

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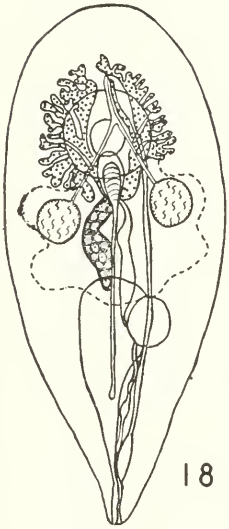

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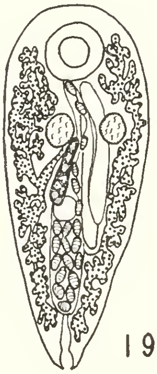

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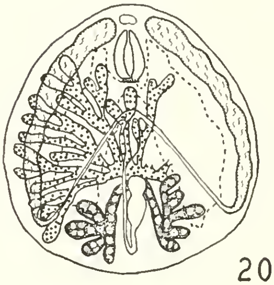

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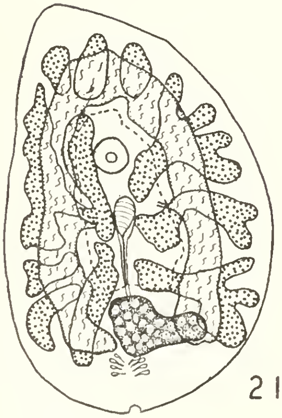

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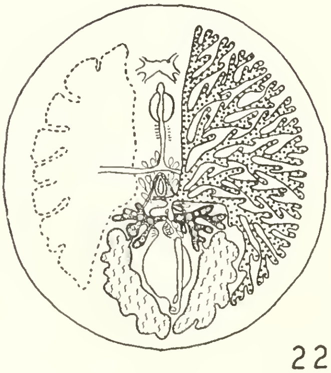

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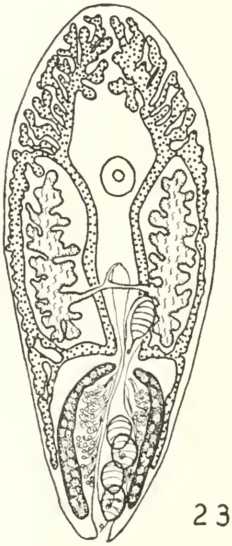

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fairly compact, not sharply delimited, rarely (*Desmote, Bicladus*) in two extensions. Brain well developed. Testes paired or unpaired, for the most part posterior to the pharynx; spermiducal vesicle present, male copulatory organ with or without cuticularized structures. Ovaries single or paired, posterior to testes and vitellaria, a single exception (*Bicladus*). Vitellaria generally extensive, commonly branched and lateral, in one case (*Cleistogamia*) peribuccal. Uterus formed as a protrusion of the common genital antrum, rarely (adult *Desmote*) an independent structure. Bursal canal, ovovitelline duct, and seminal receptacle always present. Parasites in the alimentary canal or body cavity of echinoderms or (*Collastoma*) sipunculids.

Subfamily Umagillinae Wahl, 1910 (syn. Anoplodiinae v. Graff, 1913).

Body oval to elongate; intestine saccate to tubular; testes paired (fused anteriorly in *Macrogynium* ?); vitellaria follicular and branched; ovovitelline duct enters common genital antrum ventral or anterior to the common sperm duct. Parasites of echinoderms, classes Echinoidea and Holothurioidea. Type genus: *Umagilla* Wahl, 1906; includes also *Anoplodium* Schneider, 1858; *Syndesmis* Silliman, 1881; *Cleistogamia* Faust, 1924 (as redescribed by Baer, 1938); *Wahlia* Westblad, 1930; *Anoplodiera* Westblad, 1930; *Ozametra* Marcus, 1949 (= *Xenometra* Ozaki, 1932); and *Macrogynium* Meserve, 1934.

Subfamily Collastominae Wahl, 1910.

Body oval to elongate; intestine saccate; testis single, median; vitellaria smooth or slightly branched; ovovitelline duct enters the common genital antrum dorsal or posterior to the common sperm duct. Parasites of sipunculid worms. Type and only genus: *Collastoma* Dörler, 1900.

Subfamily Bicladinae Stunkard and Corliss, 1950.

Body nearly circular in outline; intestine H-shaped, a saccate lobe on either side of body; testes paired, typically posterior but may extend anteriad in *Desmote;* vitellaria extensively branched; genital pore or pores ventral, near posterior end of body; ovovitelline duct very short, enters directly into the uterus. Parasites of echinoderms (Crinoidea). Type genus: *Bicladus* Kaburaki, 1925; includes also *Desmote* Beklemicheff, 1916.

Plate III

Figure 14. *Pterastericola fedotovi* after Beklemicheff, 1916.
Figure 15. *Collastoma minutum* after Wahl, 1906.
Figure 16. *Collastoma eremitae* after Beklemicheff, 1916.
Figure 17. *Collastoma monorchis* after Dörler, 1900.
Figure 18. *Cleistogamia holothuriana* after Baer, 1938.
Figure 19. *Cleistogamia loutfia* (Khalil and Azim, 1937) after Khalil and Azim.
Figure 20. *Desmote vorax* after Beklemicheff, 1916.
Figure 21. *Macrogynium ovalis* after Meserve, 1934.
Figure 22. *Bicladus metacrini* after Kaburaki, 1925.
Figure 23. *Ozametra arbora* (Ozaki, 1932) after Ozaki, 1932.

Key to the Family Umagillidae

Subfamily Umagillinae Wahl, 1910:
1. (16) With paired ovaries 2
2. (3) Spermiducal vesicle anterior to brain, venter not ciliated ......*Wahlia* Westblad, 1930
One species, ...*W. macrostylifera* Westblad, 1930
3. (2) Spermiducal vesicle posterior to brain, body covered with cilia, ...4
4. (7) Pharynx large, near anterior end of body, ...*Umagilla,* Wahl, 1906 5
5. (6) Single egg in uterus, ...*U. forskalensis* Wahl, 1906
6. (5) Many eggs in large, "secondary uterus"...........*U. elegans* Bock in Westblad, 1926
7. (4) Pharynx small, more posterior in position, ...8
8. (9) Vitellaria extend anterior to testes,..............................*Ozametra* Marcus, 1949
One species,..............................*O. arbora* (Ozaki, 1932)
9. (8) Vitellaria posterior to testes, ...*Syndesmis* Silliman, 1881 10
10. (15) Uterus extends forward to anterior third of body, mature egg-capsule at testicular level, ...11
11. (12) Vagina long, seminal bursa confluent with seminal receptacle, ...*S. echinorum* François, 1886
12. (11) Vagina short, independent seminal bursa,..............................13
13. (14) Specimens 2–3 mm. long, from *Strongylocentrotus,* Pacific ocean, ...*S. franciscana* (Lehman, 1946)
14. (13) Specimens 1–1.5 mm. long, from *Centrechinus,* Gulf of Mexico,....*S. antillarum* n. sp.
15. (10) Uterus in posterior third of body, extends forward only to ovarian level, ...*S. dendrastrorum* n. sp.
16. (1) With single ovary,..............................17
17. (18) Ventral surface not ciliated, ...*Anoplodiera* Westblad, 1930
One species, ...*A. voluta* Westblad, 1930
18. (17) Body entirely covered with cilia,..............................19
19. (20) Testes united anteriorly (?), ...*Macrogynium* Meserve, 1934
One species, ...*M. ovalis* Meserve, 1934
20. (19) Testes separate, ... 21
21. (24) Pharynx very large; testes small, near pharynx........*Cleistogamia* Faust, 1924 22
22. (23) Vitellaria peribuccal; single egg in uterus, ...*C. holothuriana* Faust, 1924
23. (22) Vitellaria extend along sides of body; many eggs in uterus, ...*C. loutfia* (Khalil and Azim, 1937)
24. (21) Pharynx small; testes large, extend from level of pharynx to ovary, ...*Anoplodium* A. Schneider, 1858 25
25. (30) Anterior portion of vagina enlarged as seminal bursa, ...
26. (29) Vitellaria extend almost length of body, ...27
27. (28) Ovary on left side, ...*A. parasita* Schneider, 1858
28. (27) Ovary on right side, ...*A. evelinae* Marcus, 1949
29. (26) Vitellaria shorter, about same extent as the testes, ...*A. gracile,* Wahl, 1906
30. (25) No seminal bursa, ...
31. (32) Testes small, in middle third of body; ovary lateral, ...*A. stichopi* Bock, 1926
32. (31) Testes large, nearly as long as body; ovary median, ...*A. mediale* Ozaki, 1932

Subfamily Collastominae Wahl, 1910:
One genus, *Collastoma* Dörler, 1900, with three species,
1. (2) Ejaculatory duct muscular, not chitinized, ...*C. monorchis* Dörler, 1900
2. (1) Male copulatory organ a long chitinized tubule 3
3. (4) Vitellaria simple elongate glands, ...*C. minutum* Wahl, 1906
4. (3) Vitellaria divided into 4–6 parallel branches in mature individuals, ...*C. eremitae,* Beklemicheff, 1916

Subfamily Bicladinae Stunkard and Corliss, 1950:
1. (2) Testes posterior to ovaries; single genital pore, ...*Bicladus* Kaburaki, 1925
One species, ...*B. metacrini* Kaburaki, 1925
2. (1) Testes anterior and lateral to ovaries; two separate genital pores in mature individuals, ...*Desmote* Beklemicheff, 1916
One species, ...*D. vorax* Beklemicheff, 1916

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RE-FERTILIZATION OF THE FERTILIZED EGGS OF THE SEA URCHIN [1]

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It has up to now been believed that spermatozoa cannot penetrate into the fertilized eggs of the sea urchin even if the fertilization membrane has been removed. Loeb says (1916, p. 85) on this point: "It is well known that if an egg is once fertilized it becomes impermeable for other spermatozoa." In regard to the same point, Lillie (1919, p. 161) states: "The fertilized egg does not react to fresh spermatozoa, and these do not enter it . . . and it has been repeatedly shown that removal of the fertilization membrane does not render the egg more susceptible to superimposed insemination." Just (1939, p. 190) also expresses a similar belief in his publication as follows: "With fertilization, they pass beyond the fertilizable condition. I know of only one report (Morgan, 1895), based on insufficient evidence, which claims that fertilized eggs can be re-fertilized. In my experience, eggs having been fertilized lose capacity for fertilization for neither they nor their fragments can be fertilized again."

The present author has found that when the fertilized eggs have been washed with Ca-Mg-free media after removal of the fertilization membrane, they again become permeable to other spermatozoa. In 1947, some results of the experiments were preliminarily reported by him in Japanese. More recently he has found that spermatozoa can enter the fertilized eggs without removal of the fertilization membrane if the eggs are treated with Ca-Mg-free media before the hardening of the membrane. Since these results seem to pose certain interesting problems, it should be worthwhile to report them somewhat in detail.

MATERIAL AND METHODS

The following sea urchins were employed: *Strongylocentrotus pulcherrimus, Heliocidaris crassispina, Temnopleurus toreumaticus* and *Pseudocentrotus depressus.* The egg of *Strongylocentrotus pulcherrimus* proved the best one for the investigation since the fertilization membrane can be easily removed and the clear protoplasm renders mitotic asters easily visible in the living egg.

The first insemination was performed in the usual manner. In the first series of experiments, the fertilization membrane was removed by repeatedly sucking and blowing the eggs with a pipette which had a diameter slightly exceeding that of the egg. The denuded eggs were then exposed to a Ca-Mg-free solution. After immersion for several minutes, the eggs were put back into the normal sea water and then the second insemination was carried out.

In another series of experiments, fertilized eggs were put into a Ca-Mg-free

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solution before hardening of the fertilization membrane. On their return to sea water, superimposed insemination was performed without removal of the membrane. Whether or not spermatozoa penetrated into the eggs at the time of the second insemination and afterwards took part in the cleavage was examined before and after the first or second cleavage. The re-fertilized eggs showed abnormal mitotic figures and cleavages characteristic of the polyspermic eggs.

Experimental Results

Re-fertilization of the eggs deprived of the fertilization membrane

A. Experiments in Strongylocentrotus pulcherrimus

The eggs of *Strongylocentrotus* which had been normally fertilized were deprived of the fertilization membrane mechanically. This procedure was carried out two minutes after insemination, just after the membrane had been raised and before it had hardened. A fresh sperm suspension was added to the denuded eggs thus obtained and the development of these eggs was observed. It was found that in these eggs the cleavage was quite normal and polyspermic division was as infrequent as in the control. The result of this experiment indicates that the denuded eggs in that condition cannot be fertilized again, and coincides perfectly with those of the earlier workers.

Table I

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in normal sea water and washed with Ca-Mg-free sea water (6° C.)

Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
10^{-2}	0%	1%	99%	1%
10^{-3}	0	8	92	
10^{-4}	0	82	18	
10^{-5}	1	98	1	

In the next experiments, the denuded eggs obtained by the above-mentioned procedure were washed with Ca-Mg-free sea water for 5 minutes, and were put back into the normal sea water. The second insemination was carried out in watch glasses. The undiluted sperm, taken directly from the testes of freshly opened males, was taken as a standard and various concentrations of sperm were made by ten-fold serial dilutions. To 4.5 cc. of the egg suspension was added 0.5 cc. of a sperm suspension. In all the experiments, parallel controls were run with eggs which were not inseminated again after washing with Ca-Mg-free sea water. The eggs were examined after two or three hours. One of the results of the experiments is shown in Table I.

It can be seen that more than 90 per cent of the eggs showed polyspermic features at the first cleavage if the concentration of sperm used in the superimposed insemination was 10^{-3} or 10^{-2}. Some of such eggs showed the irregular division characteristic of polyspermic eggs while the other heavily polyspermic eggs failed to divide, showing numerous asters in the protoplasm. In the control, polyspermic eggs were very few. The result indicates that spermatozoa penetrated into the previously fertilized eggs at the time of superimposed insemination and afterwards took part in the formation of the mitotic figure. The present author wishes to call this phenomenon "re-fertilization," since spermatozoa not only enter fertilized eggs but also participate in the mitotic process of the eggs.

A number of similar experiments were also conducted using a molar urea solution (pH 7.0) instead of Ca-Mg-free sea water. It was found that a molar urea solution was more effective than Ca-Mg-free sea water. One of the results is shown in Table II.

When the concentration of sperm used in the superimposed insemination was 10^{-3} or 10^{-2}, 100 per cent of the eggs were re-fertilized and heavy polyspermy occurred in high percentages. Such heavily polyspermic eggs formed many asters in the protoplasm, but they did not cleave. It appears that the formation of too many asters caused the failure of cytoplasmic division. It is also seen in Tables I and II that re-fertilization was hard to induce when the concentration of sperm in the second insemination was 10^{-5}. This is a noteworthy fact since in the usual experiments fertilization can occur with a much lower concentration of sperm.

TABLE II

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in normal sea water and washed in a molar urea solution (8° C.)

Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
10^{-2}	0%	0%	100%	1%
10^{-3}	0	0	100	
10^{-4}	0	32	68	
10^{-5}	1	96	3	

In Figure 1, microphotographs of re-fertilized eggs and controls are shown. In normal eggs polyspermy is not found (Fig. 1a). The eggs which were inseminated again after the removal of the fertilization membrane in normal sea water cleave normally (Fig. 1b). This means that re-fertilization has not taken place. In the eggs deprived of the membrane after fertilization and washed with urea solution, no irregularity of cleavage is found, though the blastomeres are spherical and well separated from each other owing to the lack of the hyaline layer (Fig. 1c). The eggs deprived of the fertilization membrane will, when washed with urea solution and inseminated again, become polyspermic (Fig. 1d). It is clear that re-fertilization has occurred in these eggs.

In a group of experiments, the removal of the fertilization membrane was done in a Ca-Mg-free medium. This method was found to be simpler and more convenient than that of the above-mentioned experiments, because the fertilization membrane was easily removable and both the removal of the membrane and washing with Ca-Mg-free medium were done at the same time. It is a well-known fact that Ca-ion is

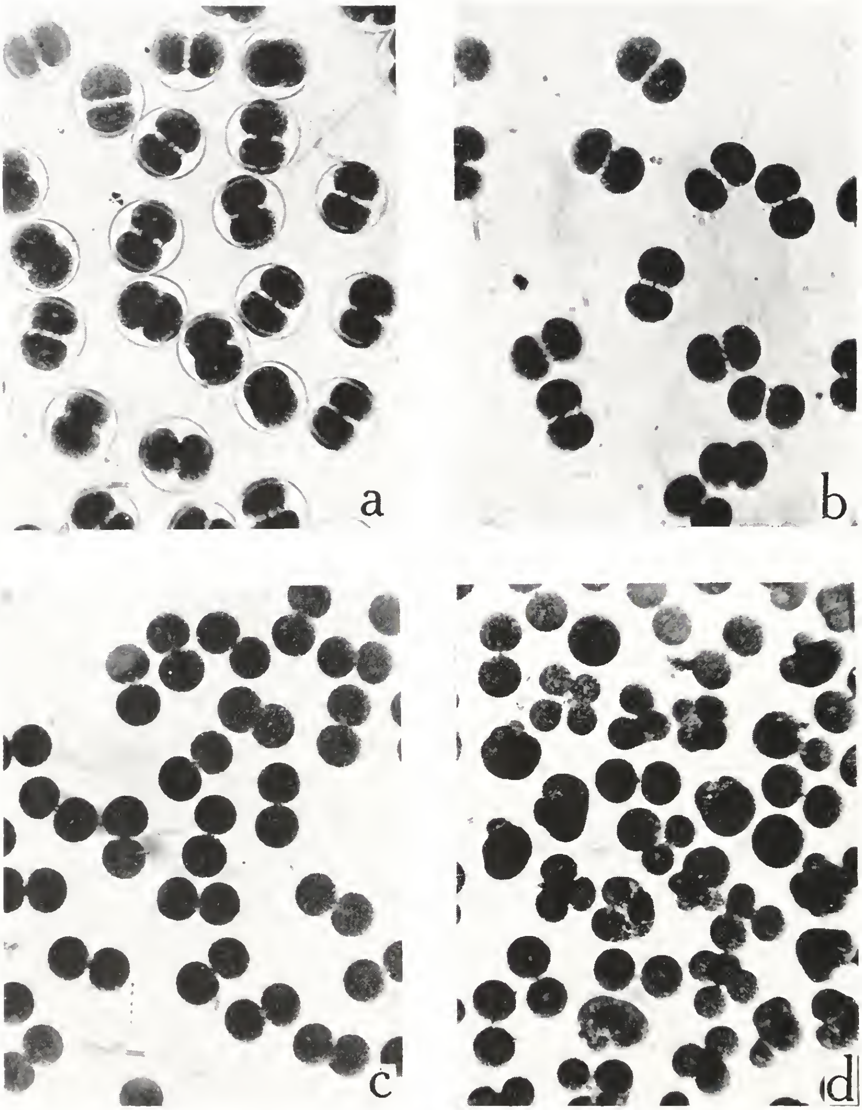

FIGURE 1. Re-fertilization of Strongylocentrotus eggs. The photographs were all taken of living eggs after the first cleavage. a. Normal eggs (control). b. Eggs which were inseminated again after removal of the fertilization membranes in normal sea water. c. Eggs which were deprived of the membrane and washed with urea solution, but not re-inseminated. d. Eggs which were deprived of the membrane, washed with urea solution and re-inseminated.

indispensable for hardening of the fertilization membrane. If the fertilized eggs are transferred into a Ca-Mg- free medium before the membrane has hardened, the membrane will not become tough and is elevated very high so that its removal is easy. One of the results is shown in Table III. It is evident from this that re-fertilization can be induced by this method.

TABLE III

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in a Ca-Mg-free medium (9° C.)

Medium in which membrane was removed	Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
		Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
Ca-Mg-free sea water	10^{-2}	1%	1%	98%	1%
	10^{-3}	0	5	95	
	10^{-4}	0	80	20	
	10^{-5}	1	98	1	
Urea solution (1 *M*)	10^{-2}	0	0	100	0
	10^{-3}	0	1	99	
	10^{-4}	0	49	51	
	10^{-5}	0	94	6	

In the above-mentioned experiments, superimposed insemination was carried out in sea water immediately after washing with a Ca-Mg-free medium. It was of some importance to ascertain whether or not re-fertilization could take place when superimposed insemination was induced at a later stage of cell division. Some experiments were carried out accordingly. The fertilized eggs were put into a Ca-Mg-free medium before the fertilization membrane had hardened, and the membrane was mechanically removed. After that they were put back into sea water in a few minutes and fresh sperm suspensions were added again at various stages of the early development of the eggs. The results are summarized in Table IV.

There is clearly indicated, by the inspection of these data, the possibility of re-fertilization taking place at any stage of the first cleavage. If the eggs are re-

TABLE IV

Re-fertilization of once-fertilized eggs at various stages of development. The membranes were removed in urea solution immediately after the 1st fertilization. (Concentration of sperm in the 2nd insemination: 10^{-3}, temp.: 13° C.)

Time from 1st insemination to 2nd insemination	Stage when observed	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs
6 m.	After 1st cleavage	1%	7%	92%
25 m.	After 1st cleavage	3	2	95
50 m.	After 1st cleavage	2	3	95
1 hr. 5 m.	After 1st cleavage	3	93	4
	After 2nd cleavage	2	8	90
2 hrs. (2-cell stage)	After 2nd cleavage	2	5	93
Control	After 2nd cleavage	2	98	0

fertilized before the anaphase, the polyspermic division takes place at the time of the first cleavage. However, if the eggs are re-fertilized after the full growth of the amphiaster, the process of the first cleavage goes on normally and polyspermic irregularity becomes visible at the second cleavage. It is of especial interest to note that the eggs are re-fertilizable even at the 2-cell stage if they have been allowed to develop in sea water after the treatment mentioned above. As shown in Figure 2, the eggs re-fertilized at the 2-cell stage become abnormal later on.

Isotonic solutions also of glycerine or thiourea are found to be effective for making the fertilized eggs re-fertilizable, while sea water containing ether, Cu-ion, trypsin, lipase, NaOH or HCl is not. Ca-Mg-free sea water becomes ineffective when any of Ca^{++}, Mg^{++}, Sr^{++} or Ba^{++} is added. It may be concluded that those media which contain any divalent cation are ineffective.

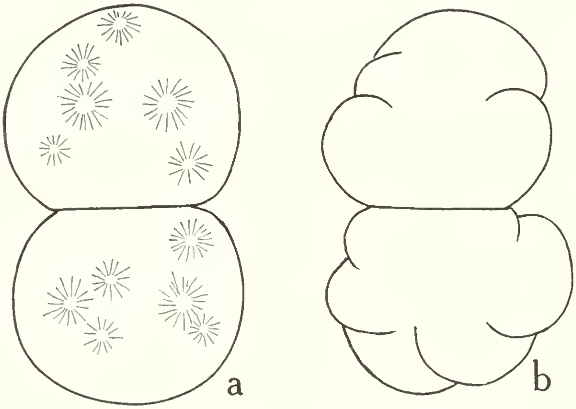

FIGURE 2. Eggs re-fertilized 5 minutes after the first cleavage. a. Thirty minutes after re-fertilization. Note many asters in each blastomere. b. One hour after re-fertilization. The second cleavage is polyspermic in each blastomere.

B. Experiments on Pseudocentrotus depressus, Heliocidaris crassispina and Temnopleurus toreumaticus

A further investigation of re-fertilization was made with three other species of sea urchins, its possibility being demonstrated to be fairly general. The three species used were *Pseudocentrotus depressus, Heliocidaris crassispina* and *Temnopleurus toreumaticus*. The procedure of the experiment was the same as was carried out with *S. pulcherrimus*. The fertilized eggs were put into Ca-Mg-free sea water and the removal of the fertilization membranes was performed in that solution. The results of the experiments, as summarized in Table V, show that all of the three species are capable of re-fertilization.

In several other experiments, Ca-Mg-free sea water was replaced with a molar urea solution with successful results.

Re-fertilization of the eggs without removal of the fertilization membrane

In recent years it has come to be recognized as a result of the works of Motomura (1941) and Runnström (1947) that the fertilization membrane is formed

Table V

Re-fertilization in several species of sea urchins. The fertilization membranes were removed in Ca-Mg-free sea water after the 1st fertilization. (Concentration of sperm in the 2nd insemination: 10^{-3})

Species	Temp.	Eggs 2-3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
		Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
P. depressus	18° C.	0%	39%	61%	0%
H. crassispina	26° C.	2	11	87	0
T. toreumaticus	26° C.	0	25	75	1

from the membrane which covers the unfertilized eggs and the material which exists below the protoplasmic surface in the unfertilized egg. On the basis of this fact it is obvious that the vitelline membrane is at first permeable to the spermatozoon but becomes impermeable as it turns into the fertilization membrane, though the so-called "wave of negativity" may have taken place earlier. For this reason, in the preceding experiments the fertilization membrane was mechanically removed before re-fertilization. The question arises as to the possibility of re-fertilization without removing the fertilization membrane when the hardening of the membrane has been inhibited. As stated before, it has been shown by several investigators that hardening of the fertilization membrane is dependent upon the presence of divalent ions such as Ca^{++} and Mg^{++} in the sea water, and lack of these ions causes a failure of hardening and thickening of the membrane (Hobson, 1932; Sugiyama, 1938a, 1938b). Accordingly an attempt was made to re-fertilize the once-fertilized eggs of *S. pulcherrimus* by the following procedure. The eggs are fertilized in the ordinary way and are put into a Ca-Mg-free medium just before the membranes begin to rise. In several minutes the membranes are elevated very high. When a molar urea solution is used the membranes become especially thin and delicate. Two to five minutes after, the eggs are put back into normal sea wa-

Table VI

Re-fertilization of once-fertilized eggs without removal of the fertilization membranes

Medium in which eggs were immersed	Temperature	Time from insemination to immersion in Ca-Mg-free medium (seconds)	Duration of immersion in Ca-Mg-free medium (minutes)	Eggs 2 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
				Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
Ca-Mg-free sea water	10° C.	30	3	0%	6%	94%	1%
		50		0	15	85	
		90		0	99	1	
Urea solution (1 *M*)	11° C.	30	2	1	2	97	0
		50		1	4	95	
		80		0	4	96	
		120		0	93	7	

ter and sperm is gently added. The membranes sink to the surface of the eggs after being returned to sea water but they are not broken during this procedure. The count of polyspermic eggs is made after the first cleavage. Some of the results are shown in Table VI.

These results suffice to show that re-fertilization occurs without any mechanical removal of the membranes if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination. Too long a stay of the eggs in sea water before their removal into the Ca-Mg-free medium causes the failure of re-fertilization since the membranes become hard enough to prevent the penetration of the spermatozoa.

DISCUSSION

It has long been accepted that fertilized eggs deprived of their fertilization membranes in normal sea water cannot be re-fertilized. This fact has been, as stated above, confirmed in the present work also. However, it has been found that fertilized eggs deprived of their membranes can, when washed in a Ca-Mg-free medium, be fertilized once again. It has also been demonstrated that when the hardening of the membrane has been inhibited, re-fertilization can take place without the removal of the membrane. In view of these findings it is justifiable to consider that in fertilized eggs there are two mechanisms of sperm exclusion. One of them is, of course, the fertilization membrane and the other is a certain substance covering the surface of the egg. The latter is so stable in sea water that it cannot be removed by any mechanical agitation. This is the reason why the removal of the fertilization membrane alone does not render the eggs re-fertilizable. However, it is noticed that this sperm-excluding mechanism becomes ineffective by washing with Ca-Mg-free sea water or non-electrolyte solutions. On the other hand, several kinds of reagents and solutions containing divalent cations were tried, but without success. These results seem to indicate that the sperm-excluding substance is soluble only in a medium which does not contain any divalent cation.

According to Just, the mechanism of monospermy in fertilization is attributed to the so-called "wave of negativity." The relationship between the wave of negativity and the sperm-excluding substance on the surface of fertilized eggs is an interesting problem. At present, however, no evidence has been furnished as to whether the sperm-excluding substance on the surface of the egg is the product of the wave of negativity or it is produced afterwards, independent of the wave of negativity.

In Urechis eggs, Tyler and Schultz (1932) have shown that the reversal of fertilization is possible by treatment with acid sea water. But with the eggs of the sea urchin no successful result has been achieved so far by means of acid sea water.

Bury (1913) reported that the fertilized eggs of the sea urchin could be fertilized again if they were exposed to a low temperature. A possible explanation of Bury's result is that the sperm-excluding substance on the surface of fertilized eggs may become ineffective at a low temperature, but a definite conclusion with respect to this point must await further investigation.

There have been several studies on the superposition of fertilization on parthenogenesis. Loeb (1913) found that the eggs of *Strongylocentrotus purpuratus,* in which membrane formation had been induced by butyric acid, could be fertilized by sperm if the membranes were torn by shaking. It was also found that the eggs of the same form were fertilizable after activation by the treatment with hypertonic sea water. C. R. Moore (1916, 1917) showed that in the case of Arbacia there was no possibility of superimposing fertilization upon parthenogenesis after optimum exposure to the activating agent. Some other workers also came to a similar conclusion, using several kinds of marine eggs. Ishida and Nakano (1947, 1950) report that if the artificially activated eggs of *Strongylocentrotus pulcherrimus* are heavily inseminated after removal of the membranes, some of them can be fertilized, and that fertilization of the activated eggs is the more easily accomplished when the removal of the membrane is performed in Ca-Mg-free solutions. The results reported here on re-fertilization correlate closely with the findings of these two authors.

Attention may be called to the fact that re-fertilization can take place at any stage of the first cleavage or even at the 2-cell stage if the eggs have been allowed to develop in sea water after the removal of the fertilization membranes and their washing in a Ca-Mg-free medium. It would be of great interest to study the possibility of re-fertilization in the later developmental stages. Unfortunately, no experimental studies have yet been made on this problem.

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SUMMARY

1. If the fertilized eggs of sea urchins (*Strongylocentrotus pulcherrimus, Pseudocentrotus depressus, Heliocidaris crassispina, Temnopleurus toreumaticus*) are inseminated again after removal of the fertilization membrane and washing with a Ca-Mg-free medium for a few minutes, re-fertilization can take place. Spermatozoa not only penetrate into the previously fertilized eggs but also take part in the formation of the mitotic figure, so that the cleavage of re-fertilized eggs is irregular and characteristic of polyspermy.

2. Re-fertilization can occur without removing the membrane mechanically if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination.

3. Re-fertilization can take place at any stage of the first cell division and even at the 2-cell stage.

4. It is justifiable to consider that there is a sperm-excluding substance on the surface of fertilized eggs. This substance is stable in sea water but is easily lost in Ca-Mg-free media.

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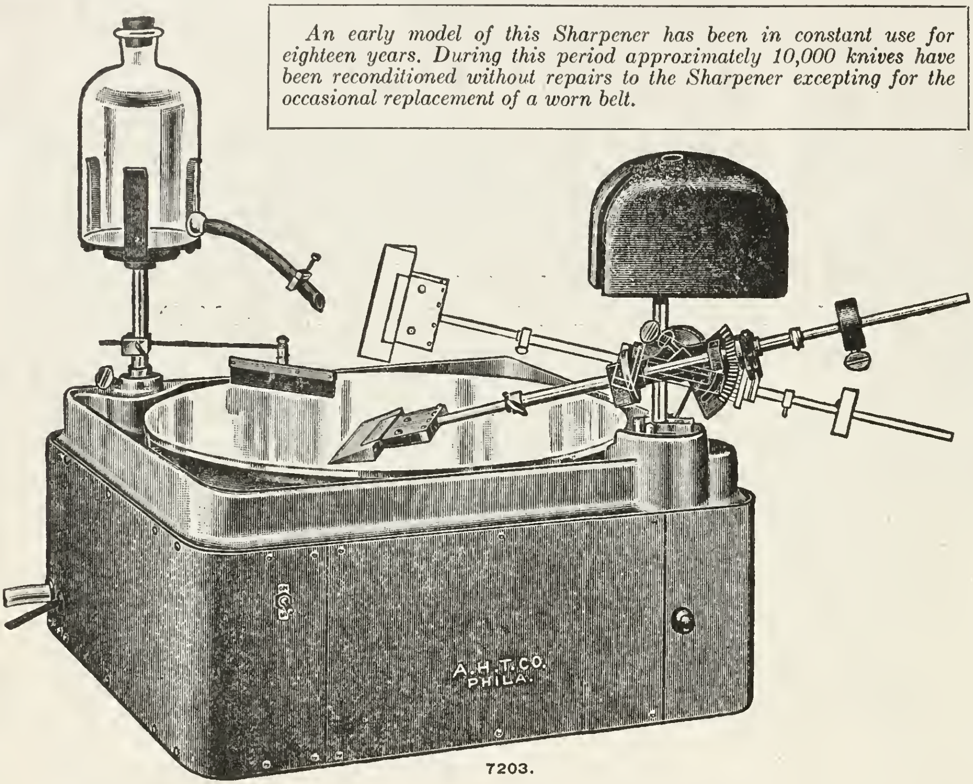

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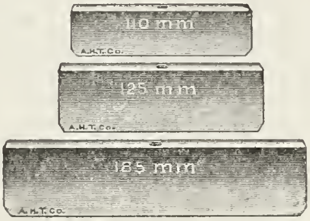

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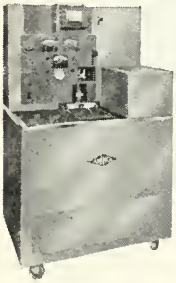

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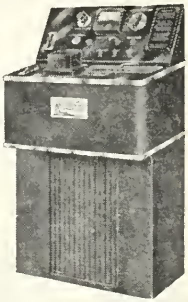

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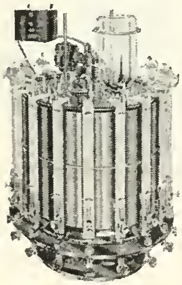

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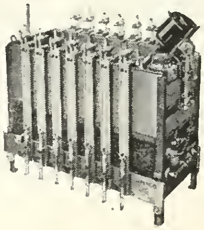

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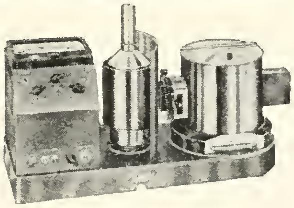

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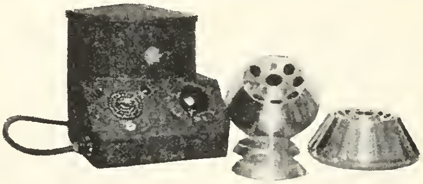

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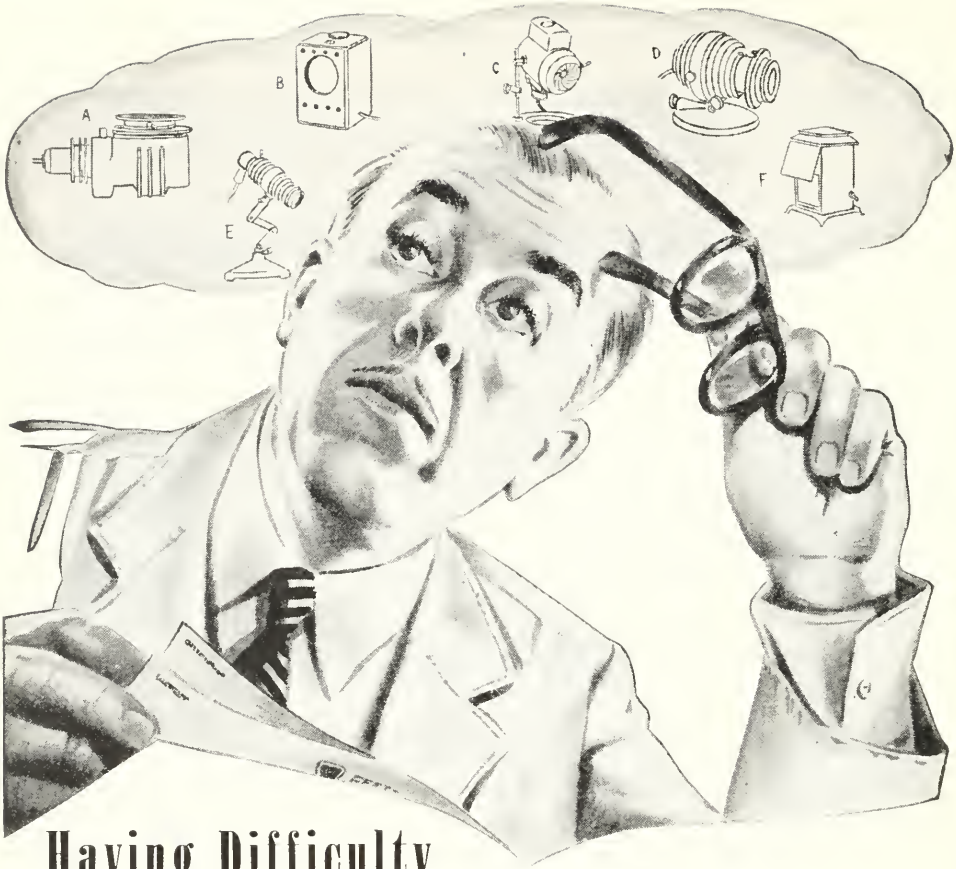

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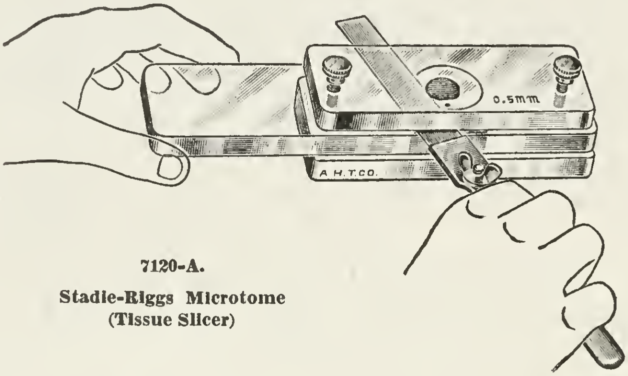

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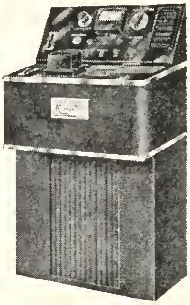

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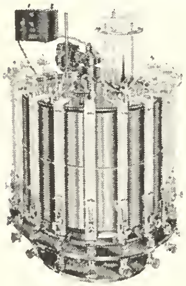

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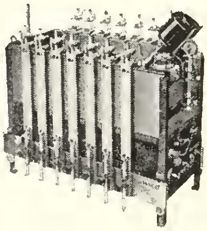

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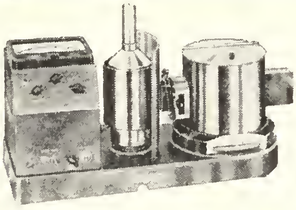

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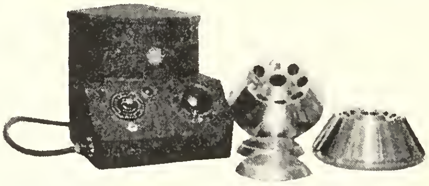

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